Stability of 21 Antihypertensive Drugs in Serum Collected in Standard (Nongel) Serum Tubes Versus Tubes Containing a Gel Separator

Solfrid Hegstad, PhD,* Olav Spigset, MD, PhD,*† and Arne Helland, MD, PhD*†

Background: Therapeutic drug monitoring of antihypertensive drugs is being increasingly used to optimize treatment and to assess nonadherence. Separator gels are often used in blood collection tubes to facilitate serum or plasma separation from other blood constituents before analyses. Drug adsorption into the separator gel presents a possible pre-analytical cause of falsely low concentrations or false negative results.

Methods: Drug-free blood from blood donors was spiked with therapeutic concentrations of 21 antihypertensive drugs, transferred to serum tubes with and without separator gel (Vacuette gel plastic tubes and plain serum plastic tubes, respectively), and centrifuged. Serum was collected immediately after centrifugation and after 24 and 72 hours of room temperature storage, samples were analyzed in triplicates using liquid chromatography–mass spectrometry.

Results: Serum samples collected immediately after centrifugation or 24 hours later, had the same drug concentrations in the gel and nongel tubes. After 72 hours of room temperature storage, verapamil and lercanidipine serum concentrations were 43% and 29%, respectively, lower in gel tubes than nongel tubes. Canrenone, diltiazem, and bendroflumethiazide showed between 10% and 20% concentration loss in gel tubes, compared with nongel tubes, with the 2 latter observed as unstable also in nongel tubes.

Conclusions: Except for verapamil, lercanidipine, and canrenone, which showed substantial concentration loss in gel tubes, gel tubes may be used for therapeutic drug monitoring purposes for the most commonly used antihypertensive drugs. Transferring serum to gel-free containers immediately after centrifugation minimizes concentration loss; however, bendroflumethiazide and diltiazem are generally unstable at room temperature.

Key Words: therapeutic drug monitoring, antihypertensive drug, pre-analytical storage, serum tube, gel separator

Antihypertensive drugs are used in the treatment of arterial hypertension, a major risk factor for cardiovascular disease. Poor adherence to antihypertensive drugs is common, often leading to treatment failure.1 Over the last decade, the measurement of antihypertensive drug concentrations in serum or other biological matrices has emerged as an objective assessment of adherence to treatment. Therapeutic drug monitoring (TDM) quantifying the concentration of a drug in serum or plasma may also be used to ensure patient dose optimization. Some evidence suggests that TDM during antihypertensive drug treatment improves adherence and blood pressure control,2 and is most likely cost-effective.3

Blood collection tubes containing separator gel are often used to collect serum or plasma for analysis. Such tubes allow for rapid serum or plasma separation from other blood constituents and are convenient in daily practice. However, during storage, drug adsorption into gel barriers may occur, as has been demonstrated for several drugs.4–8 Hence, there is concern that the use of gel separator tubes may lead to the measurement of falsely low drug concentrations or false negative results. In TDM of antihypertensive drugs, this could lead to erroneous conclusions regarding appropriate drug dosing or the assessment of the degree of adherence to therapy.

Two recently published studies demonstrated that a drug’s physico-chemical properties could influence its concentration in gel separator tubes.9,10 Lipophilic substrates such as several antidepressants and antipsychotic drugs seem to be more prone to gel barrier adsorption than hydrophilic substances. According to Steuer et al.,5 when using gel tubes, lipophilic substances with an octanol/water partition coefficient (logP) >3 and/or a “compatibility factor” (CF) >20, the latter calculated based on the drug’s logP value, polar surface area (PSA), and degree of plasma protein binding (PB), should be evaluated by stability studies.

In this study, we compared the serum concentrations of 21 antihypertensive drugs sampled in ordinary (nongel) tubes versus those sampled in gel separator tubes during a 72-hour room temperature storage period. We included the most commonly used antihypertensive drugs in Norway: beta blockers, calcium antagonists, angiotensin II receptor

Received for publication April 9, 2019; accepted August 20, 2019.
From the *Department of Clinical Pharmacology, St. Olav University Hospital; and †Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway.
The authors declare no conflict of interest.
Correspondence: Solfrid Hegstad, PhD, Department of Clinical Pharmacology, St. Olav University Hospital, 7006 Trondheim, Norway (e-mail: solfrid.hegstad@stolav.no).
Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc. on behalf of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology. This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

Ther Drug Monit • Volume 42, Number 2, April 2020
335
antagonists, angiotensin converting enzyme inhibitors, diuretics, and \( \beta \)-selective alpha blockers. The primary aim was to determine whether the use of gel tubes constitutes a significant pre-analytic source of error in antihypertensive drug concentration assessment. We also tested the hypothesis that a drug’s \( \log P \) value and \( \text{CF} \) could reliably predict the extent of concentration loss in gel tubes.

**MATERIALS AND METHODS**

**Chemicals**

Atenolol, carvedilol, diltiazem, doxazosin, irbesartan, lercanidipine, metoprolol, and pranopanolol were obtained from Sigma Aldrich (St. Louis, MO). Amlodipine, bendroflumethiazide, bisoprolol, candesartan, enalaprilat, hydrochlorothiazide, lisinopril, nifedipine, valsartan, and verapamil were obtained from Toronto Research Chemical Inc (TRC, Toronto, ON, Canada). Canrenone, losartan carboxylic acid (E-3174), and ramiprilat were purchased from Alsachim (Strasbourg, France). Amlodipine-\( d_4 \), bendroflumethiazide-\( d_3 \), bisoprolol-\( d_5 \), candesartan-\( d_5 \), canrenone-\( d_8 \), diltiazem-\( d_3 \), doxazosin-\( d_7 \), enalaprilat-\( d_5 \), hydrochlorothiazide-\( ^{13} \text{C}_2 \), irbesartan-\( d_4 \), lercanidipine-\( d_3 \), metoprolol-\( d_7 \), nifedipine-\( d_6 \), ramiprilat-\( d_8 \), valsartan-\( d_8 \), and verapamil-\( d_8 \) were purchased from TRC (Toronto, Canada). Atenolol-\( d_5 \), carvedilol-\( d_4 \), and propranolol-\( d_7 \) were obtained from Chiron (Trondheim, Norway), and lisinopril-\( d_5 \) and losartan carboxylic acid-\( d_4 \) from Alsachim (Strasbourg, France).

Liquid chromatography–mass spectrometry (LC-MS) grade acetonitrile, and LC-MS grade methanol and CaCl\(_2\) were obtained from Merck (Darmstadt, Germany). Formic acid for sample preparation (analytical quality) and mobile phase preparation (100% Aristar) were obtained from VWR International (Oslo, Norway).

Ammonium formate (\( \approx 99.995\% \) trace metals basis) and ammonium hydroxide solution (28%–30%) were purchased from Sigma Aldrich (Oslo, Norway). The Vacuette plain serum plastic tubes (5 mL, serum sep clot activator) and Vacuette gel plastic tubes (4 mL serum sep clot activator and olefin-based gel) were obtained from Greiner Bio-One (Kremsmünster, Austria). Human blank citrated whole blood was obtained from healthy blood donors not using any medication (St. Olav University Hospital, Trondheim, Norway).

**Experimental Set-Up**

Stock solutions of analytes were prepared in methanol, further combined, and diluted into 5 sets of working solutions. Preliminary tests showed no effect on drug concentrations if working solutions were diluted in pure water or 1% and 5% methanol. The internal standards were prepared in 20% methanol (vol/vol) in water. In general, drug solutions were prepared as previously described.\(^{11}\) The working solutions (500 \( \mu \)L of each) were used to spike blank whole blood (50 mL) with analytes, to achieve concentrations relevant for therapeutic use (Table 1). The samples were prepared as documented by Steuer et al.\(^9\) Spiked whole blood (4 mL) was either aliquoted into Vacuette plain serum plastic tubes (n = 3) or Vacuette gel plastic tubes (n = 3). Clotting was induced by adding 30 \( \mu \)L of 2 M CaCl\(_2\) to each tube, after aliquoting drug-spiked whole blood. The test tubes were allowed to clot for 30 minutes and then centrifuged at 2200g for 10 minutes. Serum from nongel tubes was transferred into polystyrene (PS) tubes after centrifugation. Serum aliquots were collected at baseline (0 hours) from all tubes. All tubes were stored at room temperature (23 ± 2°C), and another round of serum collection was done after 24 hours (day 1) and 72 hours (day 3). These storage periods were chosen to test realistic sample transportation time to the laboratory, and storage after centrifugation until analyses, for internal (hospital) and external samples, respectively. Analyzing the baseline serum allowed for the flexibility to test whether using gel tubes merely for collection and separation, and not transportation, could be a viable practice. All serum samples were stored at −20°C until analysis.

**Sample Preparation and Analysis**

Samples were prepared and analyzed following a previously validated and published method.\(^{11}\) Automatic sample preparation was performed using Hamilton ML Star obtained from Hamilton Robotics AB (Bonaduz, Switzerland). The sample preparation (200 \( \mu \)L) included a protein precipitation (600 \( \mu \)L acetonitrile with 1% formic acid) and filtration step, using an Ostro 96-well plate obtained from Waters (Milford, MA). The eluates were collected, evaporated to dryness, and reconstituted in 100 \( \mu \)L of methanol/water (30:70, vol/vol) before injection on an Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 \( \mu \)m) column obtained from Waters (Milford, MA). The compounds were detected on a Xevo TQ-S tandem-quadrupole mass spectrometer from Waters (Manchester, United Kingdom), equipped with a Z-spray electrospray interface. Positive and negative electrospray ionization was performed in the multiple reaction monitoring mode. All analyte accuracies ranged from −13.7% to +13.2%, and intra- and interday precisions, from 1.1% to 10.5%.

**Physico-Chemical Properties**

The logP, PSA, and PB values for all analytes were retrieved from the online PubChem database,\(^{12}\) and the handbook Clarke’s Analysis of Drugs and Poisons,\(^{13}\) as well as the Summary of Product Characteristics of each drug. The CF was calculated using the formula proposed by Steuer et al.\(^9\)

\[
\text{CF} = \frac{(\log P)^2 \times \text{PB}}{\text{PSA}}
\]

**Statistical Analyses**

Independent sample \( t \)-tests were used to compare mean drug concentrations for samples stored in nongel versus those stored in gel tubes, at each time point (0, 24, and 72 hours), and the mean drug concentrations between baseline (0 hours after centrifugation) and 72 hours, for samples stored in gel tubes. Unequal variances were assumed if the Levene test for equality of variances showed a significant difference. Statistical testing was performed when the relative difference in concentration was >10%, defined as the minimal difference to have possible clinical significance. Associations between
TABLE 1. Mean Concentrations and Coefficients of Variance (CV) of 21 Antihypertensive Drugs in Serum Samples Stored in Gel Tubes and Nongel Tubes

| Analyte (Conversion Factor)* | Gel Tube | | | | | | Nongel Tube | | | |
|-------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
|                               | Day 0   | Day 1   | Day 3   | Day 0   | Day 1   | Day 3   | Day 0   | Day 1   | Day 3   |
| Concentration (nM)            | CV (%)  | Concentration (nM) | CV (%)  | Concentration (nM) | CV (%)  | Concentration (nM) | CV (%)  | Concentration (nM) | CV (%)  |
| Amlodipine (0.409)            | 36.9    | 1.8     | 38.2    | 2.6     | 36.3    | 1.1     | 40.5    | 2.0     | 38.0    | 3.8     | 37.2    | 1.1     |
| Atenolol (0.266)              | 448     | 3.6     | 462     | 1.2     | 441     | 1.5     | 471     | 1.9     | 450     | 1.7     | 437     | 0.7     |
| Bendroflumethiazide (0.421)   | 17.1    | 1.6     | 13.2    | 1.3     | **7.3** | 2.9     | 17.8    | 2.2     | 13.0    | 2.6     | 8.3     | 2.6     |
| Bisoprolol (0.325)            | 111     | 2.7     | 120     | 1.7     | 117     | 1.6     | 114     | 2.3     | 115     | 2.1     | 118     | 1.6     |
| Candesartan (0.441)           | 123     | 2.2     | 131     | 1.4     | 129     | 2.6     | 128     | 0.9     | 126     | 3.0     | 129     | 0.6     |
| Canrenone (0.341)             | 222     | 2.5     | 226     | 3.4     | **189** | 7.7     | 233     | 1.1     | 230     | 2.1     | 230     | 0.5     |
| Carvedilol (0.407)            | 42.2    | 2.8     | 43.4    | 5.1     | 41.8    | 2.9     | 43.9    | 2.0     | 43.3    | 3.0     | 44.2    | 0.6     |
| Diltiazem (0.415)             | 90.0    | 2.6     | 86.5    | 2.9     | **58.6**| 6.5     | 94.3    | 0.8     | 86.4    | 2.6     | 72.6    | 1.9     |
| Doxazosin (0.452)             | 104     | 4.4     | 103     | 7.2     | 96.9    | 4.9     | 105     | 2.5     | 103     | 2.2     | 105     | 0.9     |
| Enalaprilat (0.348)           | 132     | 2.9     | 140     | 1.3     | 131     | 1.3     | 129     | 1.2     | 128     | 3.0     | 131     | 0.7     |
| Hydrochlorothiazide (0.297)   | 102     | 2.5     | 106     | 1.0     | 98.4    | 1.9     | 106     | 1.1     | 102     | 2.6     | 98.2    | 0.5     |
| Irbesartan (0.429)            | 1224    | 2.6     | 1292    | 2.2     | 1243    | 2.9     | 1272    | 1.6     | 1233    | 2.8     | 1205    | 0.6     |
| Lercanidipine (0.612)         | 2.50    | 13.3    | 2.16    | 6.5     | **1.74**| 11.5    | 2.46    | 11.9    | 2.25    | 3.5     | 2.46    | 1.0     |
| Lisinopril                    | 73.1    | 2.9     | 68.6    | 1.9     | 69.9    | 1.0     | 74.9    | 4.4     | 71.6    | 2.1     | 73.6    | 3.5     |
| Losartan carboxylic acid (E-3174) (0.437) | 276 | 3.2 | 283 | 4.4 | 288 | 1.7 | 279 | 3.3 | 275 | 1.0 | 281 | 0.5 |
| Metoprolol (0.267)            | 86.5    | 0.7     | 91.2    | 1.3     | 88.6    | 2.1     | 92.4    | 1.9     | 88.0    | 1.8     | 89.3    | 0.8     |
| Nifedipine (0.346)            | 114     | 3.2     | 129     | 3.5     | 125     | 4.7     | 126     | 13.6    | 123     | 4.6     | 136     | 4.8     |
| Propranolol (0.259)           | 77.1    | 2.2     | 80.5    | 1.5     | 75.8    | 2.9     | 80.0    | 0.6     | 78.3    | 2.1     | 80.1    | 1.1     |
| Ramiprilat (0.389)            | 23.1    | 2.0     | 24.0    | 1.0     | 23.8    | 1.8     | 24.3    | 1.8     | 23.6    | 2.5     | 23.7    | 1.2     |
| Valsartan (0.436)             | 1347    | 2.6     | 1416    | 3.3     | 1416    | 4.0     | 1399    | 1.7     | 1370    | 5.7     | 1402    | 0.9     |
| Verapamil (0.455)             | 96.3    | 2.1     | 92.9    | 4.9     | **58.5**| 13.0    | 103     | 1.2     | 100     | 2.7     | 103     | 0.7     |

Samples were analyzed in triplicates at each time point. Statistically significant concentration changes exceeding 10% in gel tubes compared to nongel tubes are in bold.

*To obtain the concentration in ng/mL, multiply the concentration in nM with the conversion factor.

...the physico-chemical properties of analytes and their relative concentration loss in gel versus nongel tubes after 72 hours were assessed using Spearman rank correlation tests. Two-sided P-values < 0.05 were considered significant. Statistical analyses were performed with SPSS v23 from IBM (Armonk, NY).

RESULTS

The mean serum concentrations of 21 antihypertensive drugs after storage in gel tubes and nongel tubes are shown in Table 1. None of the analytes showed any substantial concentration differences between gel and nongel tubes at baseline (0 hours after centrifugation; day 0), or after 24 hours of room temperature storage (day 1). After 72 hours of storage (day 3), the following drugs showed > 10% lower concentrations in gel tubes, compared with nongel tubes: verapamil [−43.2%; 95% confidence interval (CI), −61.3% to −25.4%; P = 0.009], lercanidipine [−29.3%; CI, −42.4% to −16.2%; P = 0.003], diltiazem [−19.3%; CI, −28.2% to −10.4%; P = 0.004], canrenone [−17.8%; CI, −33.5% to −2.6%; P = 0.037], and bendroflumethiazide [−12.8%; CI, −17.8% to −6.2%; P = 0.005]. For the remaining analytes, the measured concentrations were stable in both types of tubes.

The changes in verapamil, lercanidipine, diltiazem, and canrenone concentrations are illustrated in Figure 1. The concentrations decreased significantly in gel tubes after 72 hours of storage, compared with the baseline, for verapamil (−39.3%; CI, −52.3% to −26.2%; P = 0.001), lercanidipine (−30.4%; CI, −55.4% to −5.6%; P = 0.027), and canrenone (−14.9%; CI, −26.3% to −3.9%; P = 0.020). No concentration loss was observed in nongel tubes, for verapamil, lercanidipine, or canrenone. Diltiazem showed significant concentration loss (P < 0.001) in both tube types, howbeit higher in gel tubes. Bendroflumethiazide showed a significant concentration loss (> 50%) in both gel and nongel tubes (P < 0.001) after 72 hours of storage.
FIGURE 1. Time-dependent changes in serum concentrations for the antihypertensive drugs lercanidipine, verapamil, diltiazem, and canrenone (the active metabolite of spironolactone) during 72 hours (3 days) of storage on gel tubes versus standard (nongel) tubes. Error bars show SD.

FIGURE 2. Scatter plots showing the correlation between physico-chemical variables [octanol/water partition coefficient (logP), plasma PB, PSA, and the CF] and the relative concentration difference between gel and nongel tubes after 72 hours of at room temperature storage for 21 antihypertensive drugs. CF is calculated as (logP)^2 × PB/PSA. The vertical dotted lines represent the suggested cut-off values for CF (3.0) and logP (20) to warrant investigation of possible gel tube effects. L, lercanidipine; V, verapamil.
In a separate test, high and low sample volumes were compared to investigate whether analyte concentration could be affected by sample volume. Serum samples were prepared as described in the material and method section. Gel tubes were stored with either 350 µL or 2 mL for 72 hours. Serum aliquots were also collected at baseline (day 0). After 72 hours of storage, the concentrations of verapamil, lercanidipine, and canrenone in the gel tubes containing 350 µL of serum were 35%, 88%, and 50% lower, respectively, compared with those containing 2 mL of serum. The concentrations of all other analytes were similar, irrespective of sample volume.

The relationship between potentially relevant physicochemical properties of the analytes, and the relative concentration difference between gel and nongel tubes after 72 hours of storage, is shown in Figure 2. No statistically significant correlations were found for logP, PSA, degree of PB, or CF. However, a weak trend toward a higher concentration difference between the gel and nongel tubes with smaller PSA, and higher logP, PB, and CF, may be inferred from the trend line slopes. Lercanidipine and verapamil, which showed the largest concentration loss in gel tubes (as indicated with the letters “L” and “V”, respectively, in Fig. 2), having logP values of 6.4 and 3.8, and CF values of 35.2 and 20.3, respectively.

**DISCUSSION**

For most antihypertensive drugs, the 72 hours serum gel tube storage had no significant influence on drug concentration. Notable exceptions included the calcium channel blockers verapamil and lercanidipine, which exhibited substantial concentration loss after 72 hours of gel tube storage. Our results also suggest a certain effect of gel tube storage on the concentration of the aldosterone antagonist canrenone (the active metabolite of spironolactone), howbeit to a smaller degree. Diltiazem and bendroflumethiazide concentrations decreased substantially in both gel and nongel tubes, confirming earlier observations that these 2 substances are generally unstable at room temperature.11

Although no statistically significant correlation was observed between drug physico-chemical properties and their concentration differences between the gel and nongel tubes, our findings are in accordance with the theoretical rationale that lipophilic and highly protein bound drugs are most affected by gel tube storage. Using a lower logP limit of 3 and a lower CF limit of 20 would identify the drugs most affected by gel tube storage. Our study had some limitations that should be acknowledged. For instance, only one concentration of each drug was tested, and it is not known whether the degree of concentration loss would be different for other concentrations. Nevertheless, the concentrations chosen were those typically seen after therapeutic usage of these drugs, and we would not expect that the results should be principally different for other concentrations within the therapeutic range. It should be noted that only one specific type of gel tube was tested, hence the results cannot necessarily be extrapolated to tubes containing other gel types. Another factor that could be more thoroughly examined is the influence of the volume stored in the gel tubes. It seems that for analytes with lipophilic properties, drug adsorption to the gel is more extensive with low serum volumes. Finally, all experiments were conducted at room temperature, and cannot be excluded that higher or lower temperatures during transport to the laboratory would affect the degree of drug loss subject to gel adsorption.

**CONCLUSIONS**

In TDM of most antihypertensive drugs, serum tubes containing separator gels may be used. For concentration measurements of verapamil, lercanidipine, and canrenone, nongel serum tubes should preferably be used, although minimal concentration loss is expected if the serum is transferred to gel-free tubes immediately after centrifugation. Diltiazem and bendroflumethiazide are unstable at room temperature, regardless of storage medium, and samples should be refrigerated until analysis.

**REFERENCES**

1. Durand H, Hayes P, Morrissey EC, et al. Medication adherence among patients with apparent treatment-resistant hypertension: systematic review and meta-analysis. *J Hypertens.* 2017;35:2346–2357.
2. Brinker S, Pandey A, Ayers C, et al. Therapeutic drug monitoring facilitates blood pressure control in resistant hypertension. *J Am Coll Cardiol.* 2014;63:834–835.
3. Chung O, Vongpatanasin W, Bonaventura K, et al. Potential cost-effectiveness of therapeutic drug monitoring in patients with resistant hypertension. *J Hypertens.* 2014;32:2411–2421; discussion 2421.
4. Karpfi J, Akerman KK, Parviainen M. Suitability of collection tubes with separator gels for collecting and storing blood samples for therapeutic drug monitoring (TDM). *Clin Chem Lab Med.* 2000;38:313–320.
5. Lima-Oliveira G, Lippi G, Salvagno GL, et al. Sodium citrate vacuum tubes validation: preventing preanalytical variability in routine coagulation testing. *Blood Coagul Fibrinolysis.* 2013;24:252–255.
6. Bush V, Blennerhasset J, Wells A, et al. Stability of therapeutic drugs in serum collected in vacuum-tainer separator tubes containing a new gel (SST II). *Ther Drug Monit.* 2001;23:259–262.
7. Dasgupta A, Dean R, Saldana S, et al. Absorption of therapeutic drugs by barrier gels in serum separator blood collection tubes. Volume- and time-dependent reduction in total and free drug concentrations. *Am J Clin Pathol.* 1994;101:456–461.

8. Dasgupta A, Yared MA, Wells A. Time-dependent absorption of therapeutic drugs by the gel of the Greiner Vacuette blood collection tube. *Ther Drug Monit.* 2000;22:427–431.

9. Steuer C, Huber AR, Bernasconi L. Where clinical chemistry meets medicinal chemistry. Systematic analysis of physico-chemical properties predicts stability of common used drugs in gel separator serum tubes. *Clin Chim Acta.* 2016;462:23–27.

10. Wollmann BM, Lunde HA, Støten LK, et al. Substantial differences in serum concentrations of psychoactive drugs measured in samples stored for two days or more on standard serum tubes versus serum tubes containing gel separators. *Ther Drug Monit.* 2018;41:396–400.

11. Gundersen POM, Helland A, Spigset O, et al. Quantification of 21 antihypertensive drugs in serum using UHPLC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2018;1089:84–93.

12. National Center for Biotechnology Information. PubChem compound database. Available at: https://pubchem.ncbi.nlm.nih.gov. Accessed April 9, 2019.

13. Moffat AC, Osselton MD, Widdop B. *Clarke’s Analysis of Drugs and Poisons.* 3rd ed. Bath, Great Britain: The Bath Press; 2004.