ELSEVIER

Journal of Chromatography A, 735 (1996) 439–447

JOURNAL OF CHROMATOGRAPHY A

Determination of morphine analogues, caffeine and amphetamine in biological fluids by capillary electrophoresis with the marker technique

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Abstract

A reliable method was sought for the fast screening and simultaneous determination of amphetamine, morphine, heroin (acetomorphine), codeine (methylmorphine) and caffeine in biological fluids and drug seizures. Capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC), with detection at 200 and 220 nm, were investigated for analytes in human serum and urine. When adequate separation was not achieved in preliminary studies with CZE, further development was focused on the MEKC method. Glycine buffer containing sodium lauryl sulfate (pH 10.5) was used for the MEKC separations. The analytes and carboxylic acids used as marker compounds could be screened by a short-capillary method in less than 2 min. In the simultaneous determination of the drugs in urine and serum a longer separation of 18 min was preferred so that all the compounds, the markers and the endogenous compounds absorbing at the detection wavelength could be adequately separated in a single run. The migration times of the compounds increased in the order caffeine, morphine, heroin, codeine and amphetamine. The repeatability of the separation was tested by using two carboxylic acids as marker compounds in the determination of the migration indices of the analytes. The relative standard deviations for the migration indices were less than 1%, which is accurate enough for the determination of the drugs in biological fluids.

Keywords: Morphine; Caffeine; Amphetamine; Heroin; Codeine; Capillary electrophoresis; Body fluids

1. Introduction

Drug abuse is a serious and growing problem, and the simultaneous screening and confirmation of drugs of abuse in body fluids is of considerable importance for the investigation of intoxications, in the identification of drug users and in the control of drug addicts enrolled in withdrawal therapy.

Narcotics and other types of drugs are usually screened by gas chromatography–mass spectrometry (GC–MS) [1–3]. High-performance liquid chromatography (HPLC) [4,5], thin-layer chromatography (TLC) [6,7] and to a lesser extent supercritical chromatography (SFC) [8] have also been used in the analysis of narcotics, and also on-line coupled LC–GC has been ap-

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0021-9673/96/$15.00
PII: S0021-9673(96)00067-2

plied in the determination of heroin and its metabolites in urine [9]. The benefits of GC–MS are the sensitivity and the possibility of identifying both the native compounds and their metabolites on the basis of their mass spectra. However, many drug substances are polar, thermally degradable of non-volatile and may be difficult to analyse by GC. Although the chemical nature of compounds is not a problem in HPLC, the separation efficiency is often poorer than in GC and detection with UV absorption is less satisfactory than the detection systems available in GC. TLC is used mainly for screening of drugs, but the sensitivity is not good enough for confirmation of substances present in very low concentrations. Capillary electrophoresis (CE) is a useful technique for the simultaneous screening of different types of drugs, e.g., β-blockers [10,11], diuretics [12] and narcotics [13–16]. Both capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) have been applied. The main advantage of MEKC over CZE is that both neutral and charged compounds and also compounds virtually insoluble in water can be separated in a single run.

A frequent drawback of CE methods in the CZE and MEKC modes is the poor repeatability of absolute migration times [17], which makes the identifications of drugs unreliable, especially in biological matrices. The repeatability of an analysis depends on several factors: ionic strength of the electrolyte, age of the capillary, previous use and treatment of the capillary, applied voltage and external capillary temperature [18]. Sometimes the repeatability of the migration times can be improved by replacing relative migration times with absolute values [19]. Relative migration times are usually calculated against the electroosmotic migration time or some internal standard. The homologous series of alkylbenzenes and alkyl aryl ketones have been introduced as retention index standards to improve the reproducibility in MEKC analyses [20]. The index calculations have been based on capacity factors (k') of the index compounds and the analytes. However, with k' values, the influence of many physical parameters (e.g., viscosity, density and diffusion) is not taken into account. If the electrophoretic mobilities of carboxylic acids are used as markers instead, the influence of these factors is included [21].

When the marker technique [21] is applied in MEKC, the net mobility of the analyte is determined by its total mobility and the electrophoretic mobility while partitioned into a micelle. If the system is not too complex, the marker technique developed for CZE can be used in MEKC. To overcome the difficulty of finding the net mobility of the marker compounds, the equation used to determine the electrophoretic mobilities of the analytes in CZE can be replaced by the equations of the indices of the marker compounds. In a two-marker system, the migration index of the first marker compound is set to 1000 (Ind_1) and the migration index of the second marker compound (Ind_2) is calculated according to the equation

$$Ind_2 = Ind_1(t_{eo}/t_2 - 1)/(t_{eo}/t_1 - 1) \quad (1)$$

where t_1 and t_2 are the migration times of the marker compounds and t_{eo} is the electroosmotic migration time. The migration indices of the analytes (Ind_x) can then be calculated by the equation

$$Ind_x = [t_1t_2(Ind_1 - Ind_2) - t_x(Ind_1t_1 - Ind_2t_2)]/t_x(t_2 - t_1) \quad (2)$$

Our aim was to develop a fast screening method for morphine analoques, amphetamine, cannabis products and caffeine and a quantitative method for their simultaneous determination in human serum and urine and in drug substances seized in illicit markets. Both the CZE and MEKC methods were tested. The two-marker technique was used to calculate the migration indices for the analytes, which were used for the identification of the compounds. The linearity and repeatability of the method were studied.

2. Experimental

2.1. Materials

Amphetamine, cannabis substance, codeine phosphate, morphine hydrochloride and heroin

from plant extract containing morphine, codeine, 3- and 6-monoacetylmorphine, acetylcodeine, noscapine and papaverine were donated to our laboratory by the Crime Laboratory of the Finnish National Bureau of Investigation (Vantaa, Finland). Ephedrine, caffeine and theophylline were obtained from the Department of Pharmacy (University of Helsinki, Finland). Glycine, sodium acetate, tricine, 3-cyclohexylamino-1-propanesulfonic acid (CAPS), sodium hydroxide, potassium hydroxide and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany), and sodium lauryl sulfate [sodium dodecyl sulfate (SDS)] from BDH (Poole, UK). All reagents were of analytical-reagent grade. Water was distilled and deionized from use.

2.2. Instrumentation and conditions

The CE instrument was a Beckman (Fullerton, CA, USA) P/ACE 2050 with System Gold software. UV detection was performed at 200 nm. The capillaries were 23 cm × 50 μm I.D. for fast screening and 67 cm × 50 μm I.D. for quantification (Composition Metal Services, Worcestershire, UK), the separation lengths being 16 and 60 cm, respectively. The separation voltages were 20 kV for fast screening and 25 kV for quantification. The samples were injected by 30 mbar pressure for 5 s. The temperature was maintained at 20°C with a liquid coolant system. The capillaries were rinsed with buffer for 3 min before each run. Components of heroin sample were identified by using a Hewlett-Packard HP 5890 A gas chromatograph coupled to an HP 5989 A mass spectrometer with operated in the electron impact (EI) mode (HP-1 column, 12.5 m × 0.20 mm I.D., 0.33 μm). The GC column temperature was programmed from 150 to 290°C, the injector and detector temperatures were 300°C, the source temperature was 120°C and the carrier gas was helium at a flow-rate of 0.95 ml/min.

2.3. Electrolyte solutions

The CZE electrolytes that were tested are listed in Table 1. The MEKC electrolytes were the same, except that they contained 0.05 *M* SDS. The pH of the electrolyte solutions was adjusted with concentrated NaOH or HCl solution, after which they were filtered through 0.45-μm filters (Millipore, Molsheim, France) and degassed before use.

Table 1
CZE buffers tested

Buffer	pH	Concentration (*M*)
Glycine	3.0–4.3	0.05
Sodium acetate	5.0–6.7	0.05
Tricine	7.4–8.8	0.05
CAPS	9.5–11	0.05
Glycine	10–11	0.01–0.07
Borate	10–11	0.05

2.4. Samples

Standard solutions of the drugs were prepared by dissolving an appropriate amount of each drug except cannabis in methanol to give a concentration of 1 mg/ml. The solutions were stored at 4°C. Samples were prepared by adding standard solutions to water, urine or serum. Drug-free urine was collected from healthy volunteers after a caffeine-free diet lasting 2 weeks and dried control serum was diluted with distilled, deionized water. To all samples 0.1 M KOH (10%, v/v) was added and urine and serum samples were diluted 1:3 (v/v) with distilled, deionized water. The urine samples were filtered through 0.45-μm Acrodisc filters (Gelman Sciences, Ann Arbor, MI, USA) and the serum samples were deprotonated with methanol and filtered.

3. Results and discussion

The separation of morphine analogues (morphine, heroin and codeine), amphetamine, cannabis compounds and caffeine in biological fluids was optimized by testing the suitability of CZE and MEKC for their simultaneous determination in spiked and pretreated serum and urine.

3.1. CZE

Separation of the mixture of drugs by CZE was investigated with different buffers at pH 3–11 (Table 1). The buffers tested were mainly organic; they were chosen because they produce lower currents than inorganic buffer solutions and thus the effect of Joule heating is minimized. The compounds could not be adequately separated by CZE. In the acidic region only one of the compounds migrated to the detector in a reasonable time (<30 min), and in the basic region the separation efficiency was not sufficient to separate all the analytes simultaneously. The CZE separation was not good enough for reliable analysis, although it might be used for determining one or two of the compounds mentioned here.

3.2. MEKC

As the resolution obtained by CZE for the determination of morphine, heroin, codeine, caffeine, cannabis compounds and amphetamine proved unsatisfactory, MEKC was investigated as an alternative method. MEKC proved superior to CZE with greater flexibility and selectivity for compounds of similar electrophoretic mobility. Cannabis compounds were excluded from the MEKC studies, because they were not stable enough over a long period.

The physico-chemical properties of both the electrolyte solution and the analytes influence the separation of the analytes. The most significant properties of the electrolyte solution are the chemical composition (ions and their concentrations, modifiers, micelles), pH and absolute viscosity. The main properties of the analytes that influence the separation efficiency are their size and charge.

SDS was added to the electrolyte solutions listed in Table 1. To optimize the electrolyte solution, we studied the effect of pH in the range 3–11 and the concentrations of buffer ions and the micelle concentration both in the range 0.01–0.08 *M*. An increase in the pH of the electrolyte improved the separation and shortened the time of analysis. In the best pH range (10–11) for the separation of the analytes, three different buffers, glycine, CAPS and borate, were tested, and glycine was found to give the lowest current inside the capillary and the best resolution. The concentrations of the buffers and the micelle affected the separation, total analysis time and peak shapes. The migration times of the analytes at low buffer concentrations (0.01–0.03 *M*) were shorter than at higher concentrations, but the peaks in the electropherogram were badly tailed at low concentrations and less so when the concentration was increased. The concentration of the micelle had a similar effect as the buffer concentration on the migration times and peak shapes. The analysis time was much shorter when the SDS concentration was near its critical micelle formation concentration (CMC, 7 m*M* in water) than when the concentration was increased. On the other hand, both the resolution and peak shapes were noticeably improved when the SDS concentration was increased. The choice of optimum buffer and micelle concentration was a compromise between analysis time and resolution. The optimized electrolyte solution of 0.05 *M* glycine and 0.05 *M* SDS at pH 10.5 (voltage 25 kV, current 32 μA) in MEKC provided sufficient selectivity for satisfactory resolution. The separation of the compounds was far better with MEKC than the CZE (Fig. 1A and B). The optimized electrolyte solution was used both in fast screening of the drugs and in their simultaneous determination.

The repeatability of the analysis was studied by applying the two-marker technique developed for CZE. The potential problems due to partitioning of the marker compounds into micelles were avoided through the use of migration indices, determined so that the calculations yielded reliable approximations of v_{eo}. Methanol was used as a neutral marker of v_{eo}. Phthalic acid and *meso*-2,3-succinic acid, added to the test mixture, were used as the marker compounds in fast screening of the analytes, and in quantitative analysis phthalic acid was replaced with ethacrynic acid to shorten the analysis time. The index of *meso*-2,3-succinic acid was set to 1000 (Ind_1) and, according to Eq. 1, the migration index of phthalic acid or ethacrynic acid (Ind_2)

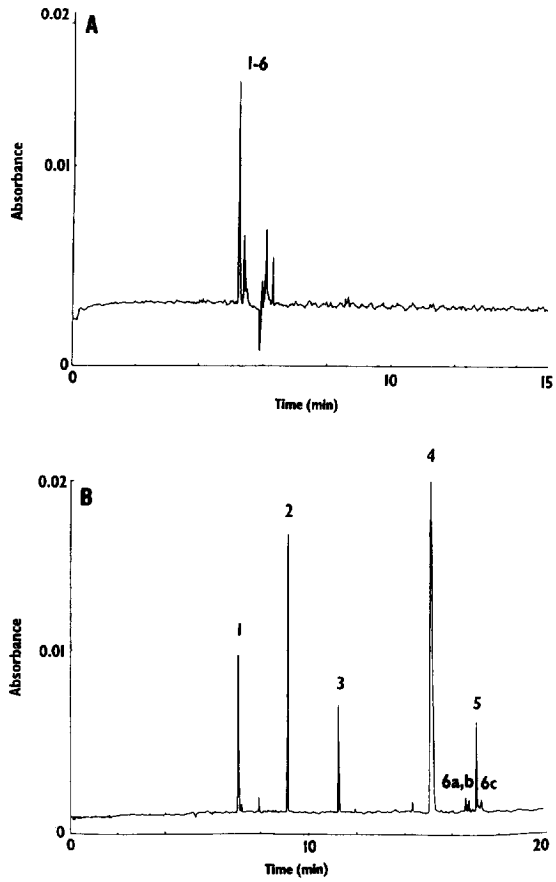

Fig. 1. (A) CZE and (B) MEKC separation of the drugs. Capillary: 67 cm × 50 μm I.D. Electrolyte solution: (A) 0.1 *M* tricine (pH 8.5) and (B) 0.05 *M* glycine–0.05 *M* SDS (pH 10.5). Peaks: 1 = caffeine; 2 = morphine, 3 = heroin; 4 = codeine; 5 = amphetamine; 6a,b and c = cannabis compounds. UV detection at 220 nm.

was 1846 and 1242, respectively. The migration indices for analytes were then calculated by using Eq. 2.

At present there is no fast CE separation method for the screening of drugs. This is a serious problem. The benefits of fast screening are obvious: valuable time is saved in routine analyses and more samples can be analysed. This is of particular importance in forensic work and in the analysis of patients' samples.

In the fast screening, very short capillaries (23 cm) were used, giving a substantial decrease in the analysis time. All the analytes migrated in less than 2 min (Fig. 2A–D). The repeatability and reliability of identification of the fast screening method were tested in nine replicate runs. The migration times and migration indices of the analytes with their R.S.D. values are listed in Table 2. The separation efficiency was also suprisingly good (Fig. 2E and F). Probably even shorter capillaries could have been used, but the capillary holder system limited the choice. The current was much higher inside the short than the longer capillary, being 117 μA when the applied voltage was 20 kV, whereas with a 67-cm capillary, the same electrolyte solution and a 25 kV voltage, the current was only 32 μA. Our method allows the fast screening of morphine analogues, amphetamine, cannabis and caffeine. With the use of short capillaries the migration times were substantially shortened and all the analytes, except heroin and codeine, were sepa-

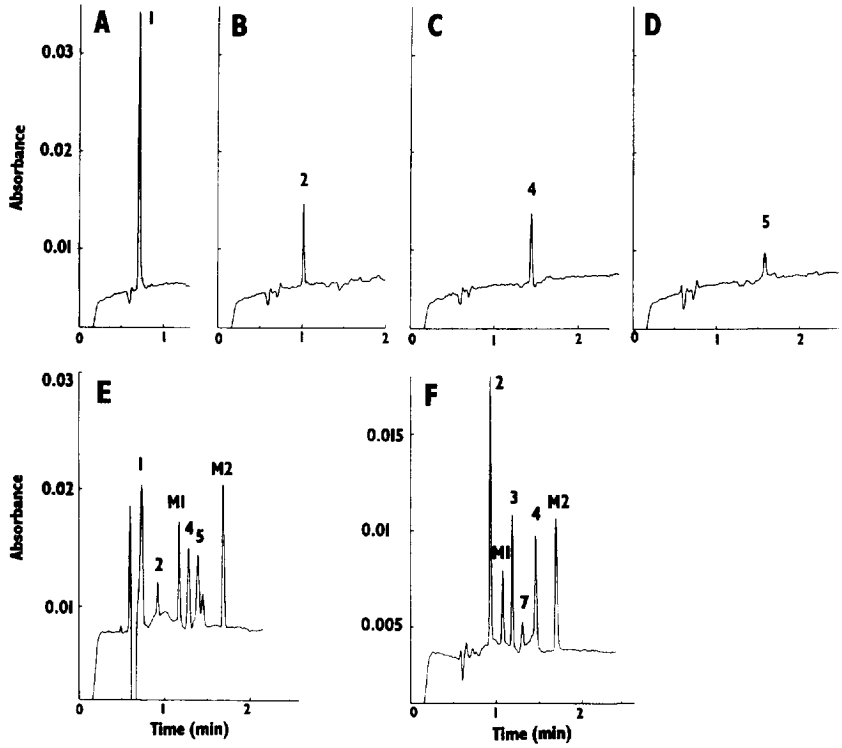

Fig. 2. Fast CE screening of (A) caffeine, (B) morphine, (C) codeine, (D) amphetamine, (E) caffeine, morphine, codeine, amphetamine and markers and (F) illicit heroin sample with markers. Capillary: 23 cm × 50 μm I.D. Electrolyte solution: 0.05 *M* glycine–0.05 *M* SDS (pH 10.5). Peaks: 1 = caffeine; 2 = morphine; 3 = heroin; 4 = codeine; 5 = amphetamine; 7 = 6-monoacetylmorphine; M1 = *meso*-2,3-succinic acid, M2 = phthalic acid. UV detection at (A) 200 and (B) 220 nm.

rated from each other. The repeatabilities of both absolute migration times (0.5–5.5%) and especially migration indices (0.2–0.7%) were good.

3.3. Quantitative analysis

To evaluate the applicability of the MEKC method to the determination of the morphine

Table 2
Migration indices and absolute migration times of the analytes (calculated from nine successive runs) with their R.S.D. values in (A) a mixture of amphetamine, caffeine, codeine and morphine and (B) illicit heroin substance

Sample	Parameter	Amphetamine	R.S.D. (%)	Caffeine	R.S.D. (%)	Codeine	R.S.D. (%)	Morphine	R.S.D. (%)
A	t_r	1.67	0.81	0.73	5.53	1.43	0.61	1.27	0.65
	Index	1678	0.51	545	0.72	1530	0.36	1401	0.18
Sample	Parameter	Morphine	R.S.D. (%)	6-MAM	R.S.D. (%)	Heroin	R.S.D. (%)	Codeine	R.S.D. (%)
B	t_r	1.08	0.50	1.31	0.38	1.19	0.42	1.46	0.33
	Index	1230	0.21	1492	0.005	1367	0.004	1619	0.006

analogues and amphetamine, we investigated the selectivity, linearity, limits of detection and quantification and the repeatability and precision of the method. In addition, the reliability of the identification of the compounds was calculated.

Human blood serum and urine contain various compounds that potentially could interfere with the separation of the analytes. However, the electropherograms did not show any interference from endogenous compounds in urine or serum (Fig. 3A–D), and drugs such as caffeine, its metabolite theophylline and ephedrine, which often are present in biological fluids, were separated well from morphine analogues and amphetamine.

Table 3 gives the linearities and the precision of the method, together with the limits of detection and quantification for the analytes. The linearity for heroin substance was studied separately because of the impurities in the heroin standard. According to stability tests, heroin in aqueous solution partially decomposes to morphine and 6-monoacetylmorphine over long sequences. Repeatabilities of the peak areas in Table 3 were calculated from six successive runs. For all compounds, except amphetamine, the repeatabilities, both intra-day (0.7–11.4%) and day-to-day (3.4–10.8%), were satisfactory.

The repeatability of the analysis was studied by using the marker technique. The absolute migration times and calculated migration indices with their R.S.D. values are given in Table 4. The R.S.D. values for the migration indices were generally less than 1%. As Table 4 shows, better repeatabilities were obtained with the migration indices than with the absolute migration times. The analyses were carried out in a temperature-controlled room, which explains why the re-

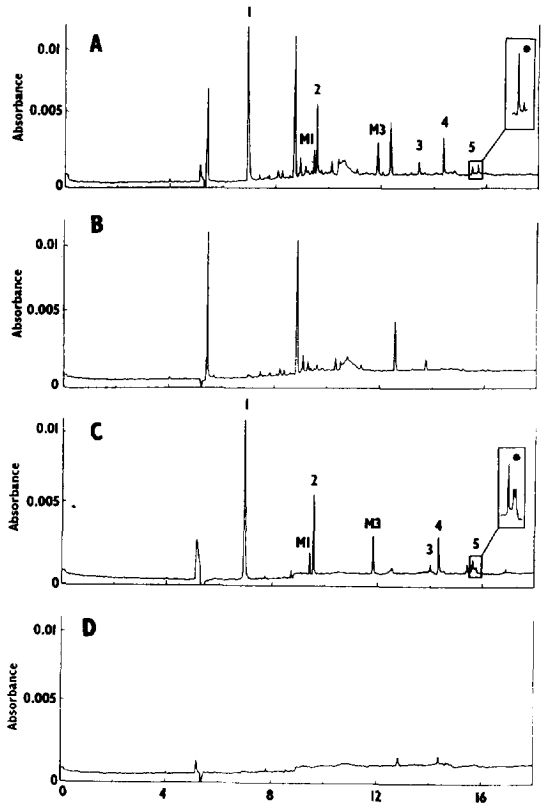

Fig. 3. MEKC separation of (A) the drugs in urine and (B) blank urine, (C) the drugs in serum and (D) blank serum. Conditions and peaks as in Fig. 2; M3 = ethacrynic acid. Insets (*): UV detection at 200 nm.

Table 3
Linearities, limits of detection (LOD, μg/ml) and quantification (LOQ, μg/ml) and repeatabilities of peak areas

Parameter	Caffeine	Codeine	Heroin	Morphine	Amphetamine
Linearity	0.998[a]	0.997[a]	0.998[a]	0.997[a]	0.990[a]
	0.990[b]	0.936[b]	0.916[b]	0.995[b]	0.951[b]
	0.963[c]	0.986[c]	0.970[c]	0.999[c]	0.987[c]
LOD	0.40	0.34	0.52	0.40	1.20
LOQ	0.50[b]	0.50[b]	0.88[b]	0.59[b]	1.60[b]
	0.50[c]	0.50[c]	0.88[c]	0.59[c]	1.60[c]
Repeatability:					
Intra-day	0.68[b]	4.73[b]	4.61[b]	3.29[b]	13.81[b]
	6.95[c]	3.13[c]	11.4[c]	1.27[c]	18.77[c]
Day-to-day	4.07[b]	5.82[b]	–	10.76[b]	15.23[b]
	3.35[c]	2.56[c]	–	4.41[c]	20.59[c]

[a] Without biological matrix.
[b] In urine.
[c] In serum.

peatability of the absolute migration times was also good; however, the migration times of the analytes in long sequences increased slowly whereas those of the indices remained constant.

4. Conclusions

MEKC was superior to CZE for the determination of the parent drug compounds amphetamine, caffeine, codeine, heroin and morphine in serum and urine. The technique was quantitative and repeatable and therefore excellent for bioanalysis, when trace fractions of drugs are analysed, but can also be used after hydrolysis step with conjugated drugs. With the help of the marker technique, migration indices could be calculated for all the compounds studied, and the use of these indices instead of absolute migration times significantly improved the reliability of identification. The rapidity of the fast MEKC method and the good separation and repeatability of the quantitative method made the technique useful for both screening and simultaneous determination of the drugs. We conclude that the migration index system can be used for the semi-identification of the drug compounds.

Acknowledgements

The authors thank the Crime Laboratory of the Finnish National Bureau of Investigation and the Department of Pharmacy of Helsinki University for the drug substances and Ordior for their cooperation.

Table 4
(A) Migration indices and (B) absolute migration times (min) of the analytes calculated from six successive runs

Parameter	Sample	Amphetamine	R.S.D. (%)	Caffeine	R.S.D. (%)	Codeine	R.S.D. (%)	Heroin	R.S.D. (%)	Morphine	R.S.D. (%)
A	Water	1457	0.07	549	0.10	1423	0.04	1217	0.12	958	0.42
	Urine	1327	0.13	443	0.14	1291	0.03	1194	0.17	956	0.01
	Serum	1537	0.55	512	0.06	1465	0.08	1242	0.11	1103	0.05
B	Water	20.0	0.30	7.7	0.20	17.5	0.41	12.8	0.33	10.4	0.58
	Urine	17.2	0.11	7.5	0.10	16.7	0.06	12.6	0.04	10.4	1.03
	Serum	17.3	0.24	7.7	2.80	18.1	4.22	12.9	0.23	10.8	2.07

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