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Method development for determining the iohexol in human serum by micellar electrokinetic capillary chromatography

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Abstract

Iohexol is widely used in clinical laboratories as a non-ionic radiographic contrast medium. Determination of its concentration in blood has a vital meaning in preventing its side effects caused by its retention in the system. A method for determining iohexol in serum by micellar electrokinetic capillary chromatography (MECC) requiring no pretreatment is developed. Electrophoresis is performed for serum samples at 25 kV with a borate buffer (50 mM; pH 9.5) containing sodium dodecyl sulfate (50 mM) and detection is carried out at 245 nm. Migration time of iohexol is 7.4 min. Linearity (0–1000 mg/l) is good and detection limit is 0.5 mg/l ($S/N = 3$). CV of intra-assay precision at a measurement concentration range of 6.2–200.1 mg/l is 1.38–4.68% and recovery rate is 96–102%. CV of inter-assay precision is 2.06–5.94% at a measurement concentration range of 10.3–155.4 mg/l. This method is characterized by determination through direct injection of serum samples of super micro-quantity into the capillary, which simplifies the determination procedure in a significant manner and improves the precision and accuracy of determination.

Keywords: Iohexol; Serum iohexol concentration; Micellar electrokinetic chromatography; Direct serum injection; Human serum

1. Introduction

Using non-ionic compounds of lower osmotic pressure and higher safety than conventional ionic compounds as contrast media for angiography is the recent trend. Development of safe non-ionic contrast media as solutions was started by Nyegaard Co., Ltd., a Norwegian company, in 1970s and, out of various compounds, iohexol was developed in 1978 as the one that best satisfied the required conditions of low toxicity, low consistency, high safety as well as liquefiability to a solution of a high concentration. This compound is non-ionic as seen from its structural formula (Fig. 1), contains a lot of hydrophilic groups and maintains high polarity. However, intravenous injection of iohexol causes serious late side effects (inclusive of shock), and aggravates symptoms of renal dysfunction in patients with deteriorated renal function or with serious renal disorder (such as anuria) by prolonging the contact of iohexol with kidney parenchyma through slowed excretion of the compound into urine. Also, special attention is called for when the compound is administered to older people who generally have lowered renal function. Iohexol, being non-radioactive,

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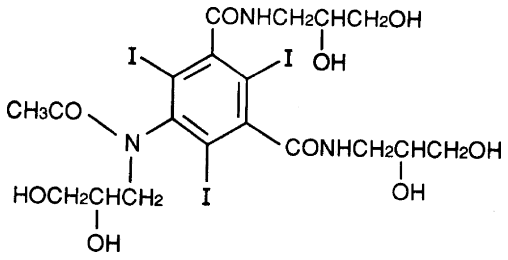

Fig. 1. Structure of iohexol.

fits in well with renal function examinations as exemplified by the fact that it is used as an indicator for glomerular filtration rate (GFR) [1–3]. In any of these cases, a simple, fast and sensitive quantification method is required so that iohexol concentration in blood can be monitored and the state of renal function can be grasped in an accurate manner. Methods for determining iohexol concentration in blood and urine using high-performance liquid chromatography (HPLC) [2,4–6] and capillary electrophoresis (CE) [1,3,7] have been reported, for all of which, specimens are used as samples for the determination after pretreatment. Therefore, these methods are not suitable for clinical laboratories because their procedures are complicated as well as time-consuming. Recently, it has been reported that CE is a useful substitute for conventional HPLC as a determination method for therapeutic drug monitoring [8]. This time, CE was used as a determination method by us with attention paid to the following advantages of CE over HPLC: (i) a fused-silica capillary tube, compared to a packed column used for HPLC, is extremely durable, and its use shortens the equilibrium time and regeneration time of a column substantially and offers higher theoretical plate number as well as higher resolution; (ii) a run buffer of comparatively simple composition is used; (iii) the system is simplified because no mechanical pump is used, requiring easier maintenance and examination of equipments; (iv) it is applicable to even patients who have difficulty in supplying enough blood for determination, because samples (serum) of only tiny volumes (several nanoliters) are required; and (v) extremely low running cost is required because only tiny volume of a run buffer is used. Thus, a simple method for determining iohexol concentration by micellar electrokinetic capillary chromatography (MECC) was newly developed by us which had a further advantage of serum samples being injected into the capillary without pretreatment.

2. Experimental

2.1. Chemical and reagents

Iohexol was provided by Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Sodium dodecyl sulfate (SDS), sodium tetraborate decahydrate and sodium hydroxide (0.1N) were purchased from Wako Pure Chemicals (Osaka, Japan). All reagents were at least of an analytical grade. Drug-free human serum was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

2.2. Instrumentation and running conditions

MECC was performed on the P/ACE^{TM} system MDQ (Beckman Coulter, Fullerton, CA, USA). Separation was carried out at 25 °C with an uncoated 67 cm (effective length 50 cm) × 75 μm ID fused-silica capillary (Beckman Coulter, Fullerton, CA, USA). A borate buffer (pH 9.5, 50 mM) containing SDS (50 mM) was used as a run buffer, whose pH was adjusted with sodium hydroxide (0.1N). This run buffer was passed through a 0.45 μm filter (Millipore, Bedford, MA, USA) and then de-aired ultrasonically for 5 min before the actual use. The capillary, after each analysis of 1 sample, was rinsed under 20 psi pressure first with a SDS solution 2% (w/v) for 5 min, secondly with sodium hydroxide (0.1N) for 3 min, thirdly with distilled water for 2 min and then conditioned with the run buffer for 5 min. Sampling time was 8 s (0.5 psi). MECC was performed at 25 kV with normal polarity and detection at 245 nm (λ_{max}).

2.3. Preparation of standard solutions

The stock standard solution of iohexol was adjusted with distilled water (20 mg/5 ml), which was further diluted with distilled water into working standard solutions of various concentrations. This stock standard solution (4 g/l) remained stable for equal or more than 6 months even when stored at 4 °C. A serum standard solution was prepared by adding the iohexol standard solution of a volume within 5% (v/v) to drug-free human serum.

2.4. Assay validation

2.4.1. Linearity

Iohexol-added serum samples of 1000, 500, 250, 125, 62.5, 31.3 and 15.7 mg/l as well as the drug (iohexol)-free human serum were measured. The results were plotted against the peak height and concentrations of iohexol to obtain linear regression.

2.4.2. The limit of quantification

An average value of concentrations measurable at a back ground noise signal-to-noise ratio of 3, and a CV value were calculated using the iohexol serum standard solution so that detection limit could be determined.

2.4.3. Reproducibility and recovery

Iohexol-added serum samples of 6.25, 12.5, 25, 50, 100 and 200 mg/l were prepared and each of them was measured five times. Recovery rate was computed by comparing each iohexol-added serum sample with the iohexol standard solution of the same concentration. To obtain inter-assay precision, iohexol-added serum samples of three different concentrations were prepared and each of them was measured on four consecutive days ($n = 2$). During this whole process, samples were stored at 4 °C.

2.4.4. Influence of endogenous substances in serum

Influence of endogenous substances in serum on the iohexol determination was evaluated by comparing the electropherogram of the drug-free human serum, which contained no iohexol, with that of the iohexol-added serum sample.

3. Results and discussion

Proteins that account for the major part of serum are troublesome existence which create an essential issue that analysis must be performed after appropriate sample preparation so that interference from proteins can be avoided. Extracting highly polar compounds such as iohexol from serum is difficult and therefore, de-proteinization is more suitable for them. Sádecká et al. [9], in their vancomycin determination, has recommended ethanol as a de-proteinizer which can be generally used, while acetonitrile has been used in most reports [1,3,7,10]. However, in de-proteinization with acetonitrile, measurement errors arising from sample evaporation or the de-proteinization process need to be compensated using appropriate internal standards [1,3,7]. In addition, although this method can be applied to analysis with a detection wavelength equal or more than 240 nm, in detection with shorter wavelength, it can make analysis more difficult due to great influence of substances such as proteins left unremoved. Shihabi and Constantinescu [7] analyzed by CE, samples prepared by diluting original serum samples with a borate buffer into a concentration 50 times as low as the original serum samples, and have reported that this method could only be applied to samples of iohexol concentrations equal or more than 1000 mg/l. Because serum protein binding of iohexol in serum is almost zero [11], using ultrafiltrated serum as a sample can be an alternative method, but it makes determination cost comparatively higher. In general, a simple and fast analysis method is desired when drug analysis is performed in clinical laboratories. Therefore, direct injection is recommended where no sample preparation is performed for serum samples [12–14]. Considering the fact that iohexol is non-ionic, MECC is believed to be the most suitable method for injecting serum directly into the capillary. Evaluation was performed on the composition of the run buffer. In terms of pH, sensitivity became the maximum when pH was 9.5, while the migration time of iohexol became longer as pH increased (Fig. 2). Thus, pH value of 9.5 was adopted in consideration

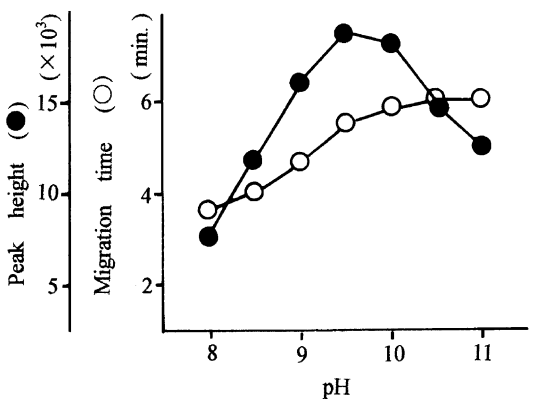

Fig. 2. Effect of pH on the migration times and peak height of iohexol. Run buffer: SDS (50 mM) in borate buffer (25 mM); sample: standard serum (100 mg/l).

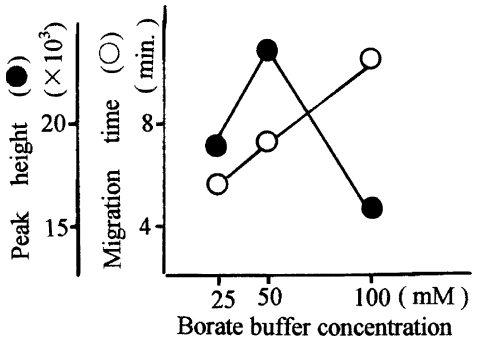

Fig. 3. Effect of borate buffer (pH 9.5) containing SDS (50 mM) concentration on the migration times and peak height of iohexol. Sample: standard serum (100 mg/l).

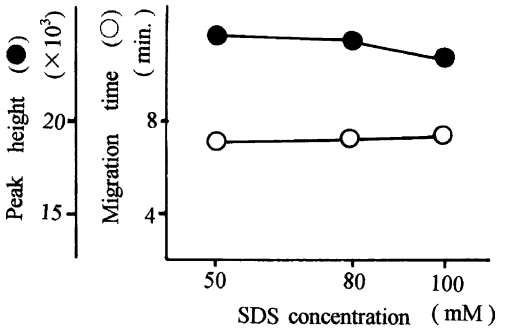

Fig. 4. Effect of SDS concentration in borate buffer (50 mM, pH 9.5) on the migration times and peak height of iohexol. Sample: standard serum (100 mg/l).

of a peak shape, etc. In evaluating the concentration of the borate buffer, the maximum sensitivity was obtained at 50 mM and the migration time became gradually longer as ionic strength increased (Fig. 3). Garcia and Shihabi [15], in determining theophylline in serum, have reported that, the higher the molarity of the run buffer became, the more precision and accuracy of determination improved. On the other hand, in our evaluation (in determining iohexol), when a 100 mM borate buffer was used, the value of the electric current running in the capillary became about 290 μA, which was undesirable. Thus, a 50 mM borate buffer was used for this method. In the evaluation of SDS concentration (50–100 mM), the migration time became 7.22 min at a SDS concentration of 50 mM, 7.34 min at 80 mM and 7.44 min at 100 mM, showing a slight but certain increase of the migration time according to the increase of the SDS concentration, while a change in sensitivity was hardly observed (Fig. 4). Thus the 50 mM SDS was selected for use. In evaluating load voltage (Fig. 5), substantial prolongation in migration time was observed as the load voltage decreased and an iohexol peak did not appear under 10 kV even after 30 min elapsed, though it is not shown in the figure. Therefore, 25 kV was adopted in order to achieve faster analysis. Fig. 6 shows the electropherograms of the drug-free human serum and iohexol-added serum. Good separation was achieved with no interference from endogenous substances in serum on the determination. Linearity of 0–1000 mg/l was good ($r = 0.9999$). Detection limit was 0.5 mg/l at a signal-to-noise ratio of 3. When 250 mg/kg (patient's weight) and 500 mg/kg (patient's weight) of iohexol were administered to patients, respectively, the iohexol concentration in serum decreased gradually as time elapsed and its concentration range became about 1–8700 mg/l [16]. This means this method had enough sensitivity to cover this concentration range. Table 1 shows test results of intra-assay precision, recovery rate and inter-assay precision. As for the results of iohexol analysis by CE after de-proteinization of serum samples with acetonitrile, Jenkins et al. [3] have reported CV of intra-assay precision to be 4.3–10.8% (20–80 mg/l), while Rocco et al. [1] have reported the same to be 2.7%, CV of inter-assay precision to be 5.1% and recovery rate to be 95–105%. Shihabi and Constantinescu [7] have reported CV of intra-assay precision to be 2.7% with the peak height at a concentration

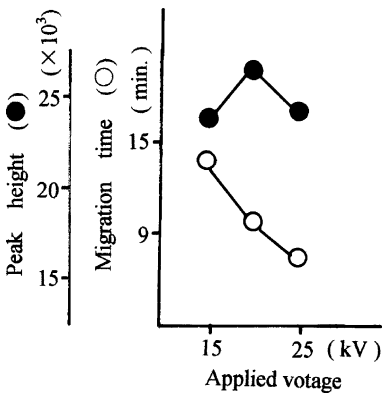

Fig. 5. Effect of applied voltage on the migration times and peak height of iohexol. Run buffer: SDS (50 mM) in borate buffer (50 mM, pH 9.5); sample: standard serum (100 mg/l).

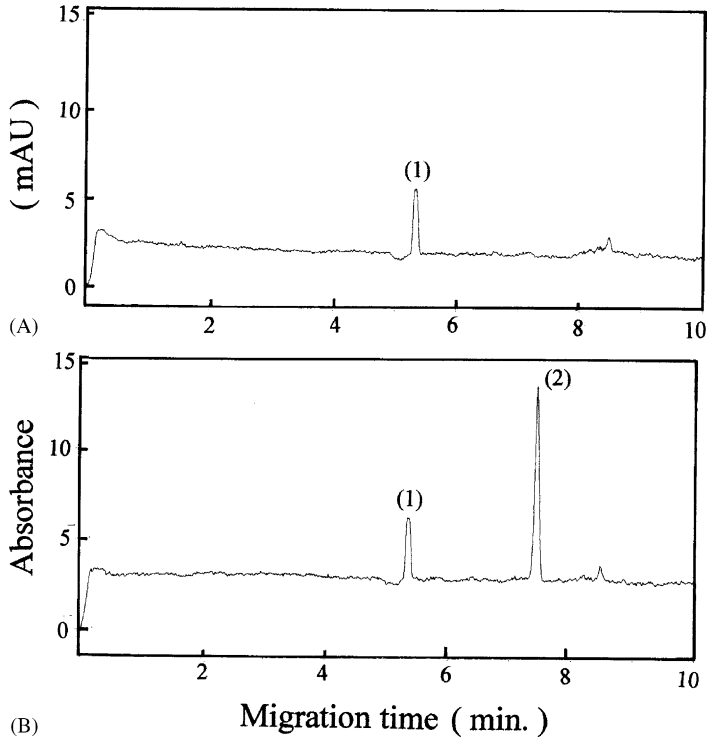

Fig. 6. Typical chromatograms of a blank serum (A), a serum standard spiked with iohexol (42.7 mg/l) (B). (1) Unknown substance; (2) iohexol.

of 50 mg/l and similarly, CV of inter-assay precision to be 5.1% at 100 mg/l. Therefore, the precision test results of this method were satisfactory as well as enough applicable to clinical laboratories. Moreover, as this method employs direct serum injection, there is no problem as far as recovery rate is concerned. As for analysis time, migration time of iohexol has been reported to be 2.6–5.25 min [3,7], and judging from this, 7.4 min of its migration time obtained by this method seems a little too slow. However, this can be said to be rather fast compared to the reported results when the fact is considered that no sample preparation is performed with this method. In addition, in this method, a 15 min rinsing process of the capillary is given after each determination of one sample. This rinsing process is longer than the migration time of iohexol and such a careful washing of the capillary helped to achieve good precision in a stable manner.

Table 1
Intra-assay and inter-assay precision and recovery of spiked serum samples for iohexol assay

Concentration (mg/l)	Precision (%)		Serum recovery[a] (%)
	Intra-assay[a]	Inter-assay[b]	
6.3	3.23	–	98.4
10.3	–	5.94	–
12.5	4.68	–	96.0
25.0	1.76	–	102.0
38.8	–	5.36	–
50.0	1.70	–	99.6
100.0	1.38	–	101.0
155.4	–	2.06	–
200.0	3.15	–	100.0

[a] Based on $n = 5$.
[b] Based on $n = 2$ for 4 days.

4. Conclusions

This method is a specific and highly accurate method for determining iohexol concentration by MECC through direct injection of serum into the cap-

illary, much improved in determination time as well as simplified in procedure even when compared with conventional CE which employs sample preparation.

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