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Stepwise binary gradient high-performance liquid chromatographic system for routine drug monitoring

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Abstract

Drug therapy is usually optimized by concentration measurement in patient serum. High-performance liquid chromatography (HPLC) is one of the most important analytical techniques used for therapeutic drug monitoring (TDM) of drugs for which no immunoassay kits are available. HPLC has been frequently used for screening purposes in toxicology, too. The Merck Tox Screening System (MTSS) has been developed for the identification of substances by a combination of gradient HPLC with diode-array detection and identification with a database system. For routine TDM an isocratic HPLC system is more suitable because of shorter analysis time, better reproducibility of retention index and better precision of results. Therefore we defined a set of methods in steps of 10% of the two MTSS eluents. Three examples are shown: Amiodarone, Indometacine and Thiopental. New applications to test for other substances can be transferred to an isocratic system after a complete MTSS gradient run.

Keywords: Drug monitoring; Amiodarone; Indometacine; Thiopental; Mefenamin; Thiobutabarbital; Promazin

1. Introduction

Today numerous drugs are available, and optimization of therapy is usually carried out by concentration measurement in patient serum. High-performance liquid chromatography (HPLC) is one of the most important analytical techniques used for routine therapeutic drug monitoring (TDM) of drugs for which no immunoassay kits are available. One of the advantages of HPLC in comparison to gas chromatography (GC) is the simple sample pretreatment. For samples of human materials reversed-phase columns are preferred because of water-based mobile phases can be used, and because of ease of equilibration, and broad applicability.

Formerly, thin layer chromatography (TLC) of urine samples was the only method generally used for the detection of overdose and abuse of unknown drugs. For exact identification of a drug it could be necessary to use different TLC systems; in systematic screening studies a combination of up to 12 TLC systems has been proposed [1]. Combined gas chromatography–mass spectrometry (GC–MS) is believed to be the best method for the analysis of unknown analytes because of its high separating efficiency and its applicability to trace analysis. This method, however, is rather time-consuming and needs derivatization of the sample extract. HPLC has also been frequently used for toxicological screening purposes. Combination of HPLC with UV spectrometry has advantages over one-dimensional detection methods such as UV or fluorescence detection [2], but it does not have the discriminating power of

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GC–MS, which is still used as reference technique [3].

The Merck Tox Screening System (MTSS) has been developed for the identification of substances by using one or more analytical techniques. The database contains data for five different TLC systems, one GC system, and a HPLC system; it contains retention index (I) data obtained with a gradient reversed-phase system at fixed temperature and diode-array spectra [4]. The MTSS program generates a list of substances that qualify for identification with a corresponding similarity index.

For routine TDM an isocratic HPLC system is more suitable because of its shorter analysis time, better reproducibility of I-data and better precision of results. Different reversed-phase columns, buffers, and mixtures of organic solvents are used in different applications for HPLC determination of drugs. Changing from one application to the next would mean long equilibration times for the columns, however. Therefore we decided to use a standardized system, based on identical columns and only two mobile-phase components, i.e. triethylammonium phosphate buffer and acetonitrile. These compounds are also used in the MTSS gradient separation system from which the elution sequence of the drugs can be transferred to an isocratic system. To this end we defined a method with 10% concentration steps of the two MTSS eluents.

2. Material and methods

The HPLC system consisted of a Merck Hitachi L-6200 intelligent pump with ternary gradient and mixing chamber. Detection was performed with a Merck Hitachi L-4500 diode-array detector (DAD). A WO Electronics M-Jet Column oven was set at 25°C. A LiChroCART 125-4 HPLC cartridge with Lichrospher 60 RP Select B (5 μm) was chosen as separating column and LiChroCART 4-4 HPLC cartridge with Lichrospher 60 RP Select B (5 μm) as guard column (all components from Merck, Darmstadt, Germany).

The two MTSS eluents were triethylammonium phosphate buffer, pH 3.0, 0.025 mol/l (Fluka, Buch, Switzerland; eluent A), and acetonitrile CertiFi grade (Fisons, Loughborough, UK; eluent B). In-house-purified water was used for cleanup procedures (eluent C). To avoid bacterial growth eluent A was altered to 98% buffer and 2% acetonitrile.

Degassing of solvents was performed by ultrasonic treatment.

The standard operation procedure for gradient elution of the MTSS system is defined as follows: gradient, 0 min 100% A; 30 min 30% A and 70% B; 33 min 100% A; 43 min 100% A for equilibration prior to the next run.

Calibrators and controls were prepared by mixing pool sera with dissolved pure substances.

Sample preparation was done by precipitation of 100 μl calibrator, control or patient serum with 200 μl acetonitrile and internal standard (I.S.), 5 min centrifugation (12 000 g) and direct injection of 50 μl of the supernatant. Quantitation was performed by the internal standard method.

Sensitivities were calculated for a signal-to-noise ratio of 4.

3. Results

For routine application of a drug first a complete gradient MTSS run is performed. Depending of the observed retention time one of the 10%-steps of the eluent A/eluent B mixture is chosen. A suitable internal standard with a similar extraction behaviour is then selected from the retention time list of the MTSS gradient system and an isocratic step run is done on the gradient HPLC system. The optimal wavelength for UV detection of both drug and internal standard is determined from the UV spectra of the DAD. The tested application is transferred to a low-cost isocratic HPLC system with RP Select B as analytical column and UV detection. The advantage of this detector is its higher sensitivity compared to the DAD. The eluent is recycled to save solvent. In the following we show three examples of such isocratic HPLC applications with the observed retention times (t_R).

3.1. Amiodarone

The isocratic system was eluent A–eluent B (10:90). The flow-rate was 1 ml/min. A commercial calibrator/control set containing Amiodarone and

desmethyl-Amiodarone was used for calibration (high level, 3 mg/l each) and quality control (mid level, 1 mg/l each; DMD, Gailingen, Germany). Detection was performed at 254 nm. Promazin (5 mg/l in acetonitrile) was used as I.S. and precipitating reagent (see Fig. 1). Sensitivity was calculated to be 0.09 mg/l. The system was linear to at least 20 mg/l Amiodarone. The coefficient of variation (n= 10; day-to-day) was 4.9% for the 1 mg/l control.

3.2. Indometacine

The isocratic system was eluent A–eluent B (40:60). The flow-rate was 1 ml/min. Detection was performed at 280 nm. The calibrator and control contained 2 mg/l and 1 mg/l Indometacine in pool serum, respectively. Mefenamin (2 mg/l in acetonitrile) was used as I.S. and precipitating reagent (see Fig. 2). The sensitivity was calculated to be 0.11 mg/l. The system was linear to at least 20 mg/l Indometacine. The coefficient of variation (n=10; day-to-day) was 3.2% for the 1 mg/l control.

3.3. Thiopental

The isocratic system was eluent A–eluent B (50:50). The flow-rate was 2 ml/min. Detection was performed at 283 nm. The calibrator and control contained 10 mg/l and 15 mg/l Thiopental in pool serum, respectively. Thiobutabarbital (10 mg/l in acetonitrile) was used as I.S. and precipitating reagent (see Fig. 3). The sensitivity was calculated to be 0.23 mg/l. The system was linear to at least 100 mg/l Thiopental. The coefficient of variation (n=10; day-to-day) was 2.9% for the 15-mg/l control.

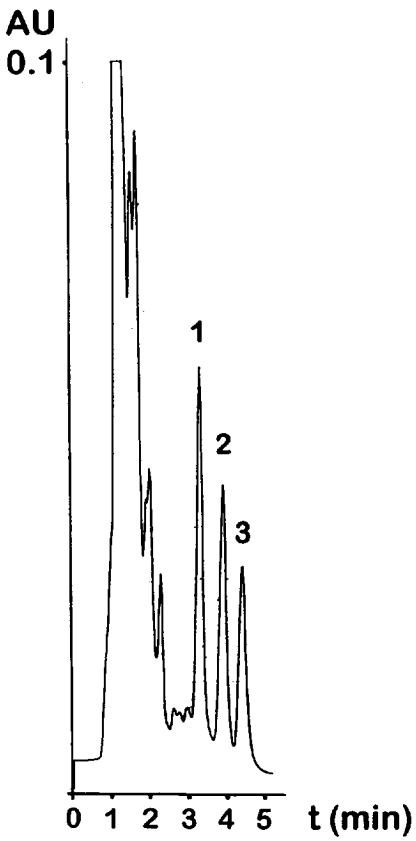

Fig. 1. Amiodarone. Mobile phase, eluent A–eluent B (10:90); flow-rate, 1 ml/min; detection, 254 nm; Promazin as I.S. t_R: Promazin (1) 3.39 min; desmethyl-Amiodarone (2) 4.06 min; Amiodarone (3) 4.58 min.

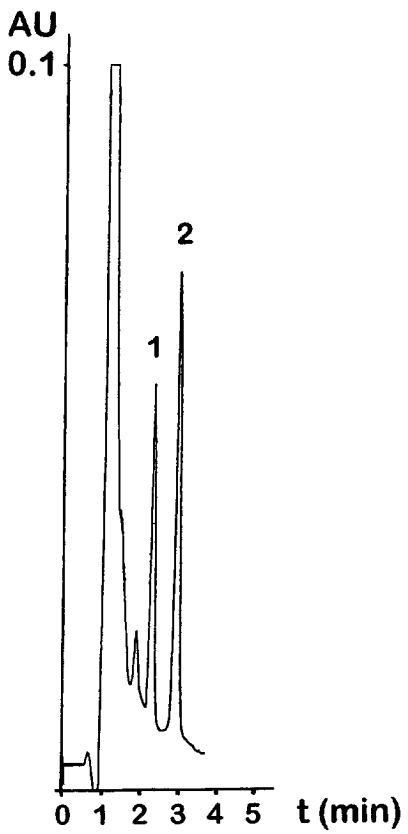

Fig. 2. Indometacine. Mobile phase, eluent A–eluent B (40:60); flow-rate, 1 ml/min; detection, 280 nm; Mefenamin as I.S. t_R: Indometacine (1) 2.28 min; Mefenamin (2) 2.94 min.

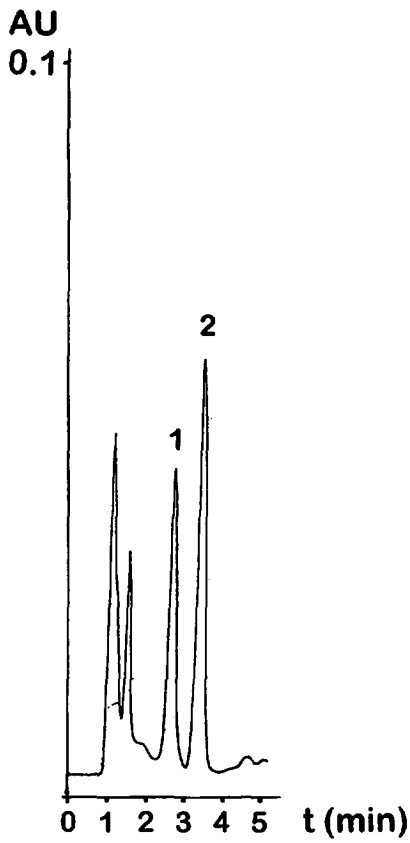

Fig. 3. Thiopental. Mobile phase, eluent A–eluent B (50:50); flow-rate, 2 ml/min; detection, 283 nm; Thiobutabarbital as I.S. t_R: Thiobutabarbital (1) 2.70 min; Thiopental (2) 3.46 min.

4. Discussion

The above stepwise isocratic systems each have an analysis time of 5 min each, which is considerably shorter than the analysis times of the gradient system (43 min). For higher throughput of samples different step mixtures of the two eluents can be applied to parallel the low-cost isocratic HPLC systems with RP Select B as analytical column with improved column-to-column reproducibility and UV detection. Such applications can also be performed on a single HPLC system with a binary gradient solvent delivery system. Between the different solvent compositions it is preferable to perform a cleanup, consisting of gradients of 0–2 min 100% C, 6 min 100% B, 10 min 100% A to obtain shorter equilibration times.

Every new test for unknown substances is done in the same way as above. First, a complete MTSS gradient run is performed and one of the stepwise linear HPLC systems is selected. Then an internal standard is chosen; preferably the I.S. used in the selected isocratic system is employed in order to reduce the number of precipitating reagents. If this is not possible, an other internal standard with similar chromatographic behaviour (*I*), similar cleanup procedure (acid or basic extraction with extraction cartridges or protein precipitation with acetonitrile), and a similar detection wavelength has to be selected. With a set of 10 mixtures of two solvents (0% B to 90% B) most of the routinely used drugs can be quantitated even on a single and simple HPLC system.

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