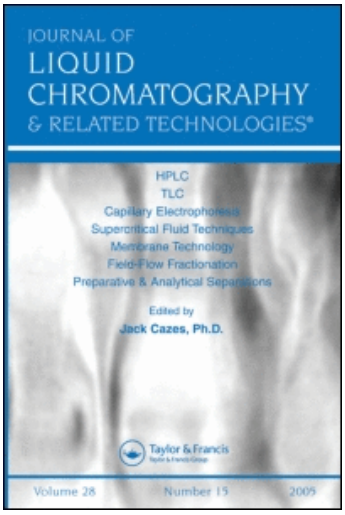

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AN AUTOMATED ANALYTICAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR IOPAMIDOL SOLUTIONS USING A BENCHMATE WORKSTATION

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ABSTRACT

A flexible compact robot system for sample work-up and preparation of calibration standards for HPLC analyses is presented. The equipment consisting of a BenchMate workstation and a conventional HPLC system was used to analyse aqueous solutions containing iopamidol, a contrast medium. The analytical procedure was characterized with respect to sensitivity, selectivity and linearity of the detector response. The use of the workstation yielded excellent data with respect to accuracy and intra-assay precision. The application could be used after few modifications to determine other compounds in solution or microcrystalline suspensions. The automatization of this kind of analysis saved man-hours (ca. 25%) and could reduce the costs of glassware and of solvents. An additional advantage is the reduced individual exposition to solvent vapour and other chemicals.

INTRODUCTION

Within the framework of drug research and development compounds are characterized toxicologically and pharmacokinetically. The formulations, e. g. solutions, microcrystalline suspensions, test-substance diet mixtures, lactose premixes and tablets, administered to the test animals are monitored for correct concentration to validate the preparation process. Additionally, stability and homogeneous distribution of the active ingredient in certain formulations are investigated. Numerous compounds occur in similar concentrations in formulations of the same type. Such samples are usually analysed by HPLC. Furthermore quality control units checking similar features of developmental and final market formulations to be administered in man work within the same field.

In modern laboratories the equipment for HPLC is fully automated and is often computer-controlled leading to time saving and unattended operation. However, the preparation of calibration standards and sample work-up is still done manually and consequently is tedious and time consuming. Exposition to the laboratory personnel to potentially toxic compounds and solvents is unavoidable in many cases.

There is a current trend for an increased automation in analytical laboratories, replacing many manual operations (1-3). This trend applies in particular to laboratories with a high sample turnover, as such robot systems are expensive, space consuming and require highly trained personnel. An alternative is the BenchMate Workstation (4) which is less expensive, takes up little room and is easy-to-use. Some fully or partially automated applications were reported for the determination of drugs and/or their metabolites both in plasma samples (4,5), in pharmaceutical formulations (6) and in samples obtained from in-process control (7). Analytical procedures could be automated yielding accurate and repeatable data.

The aim of our experiments was to develop generally usable procedures which should be able to replace the manual preparation of calibration standards and manual work-up of similar samples with different compounds. For the first experiments iopamiol dissolved in aqueous solutions was chosen. Iopamidol is a monomer iodine containing contrast medium for uroangiography (8,9).

EXPERIMENTAL

Instrumentation

The HPLC equipment consisted of two Model 510 pumps, a Model 680 automated gradient controller (all by Waters, Eschborn, FRG), a Perkin-Elmer ISS-100 autosampler and a Spectroflow 773 UV-detector (Kratos, Karlsruhe, FRG). The detector was connected via an interface to a mainframe computer (VAX 4000-300, Digital Equipment, Munich, FRG) for data acquisition and evaluation. The chromatograms were evaluated with ACCESS*CHROM Release 1.8 (Perkin-Elmer). UV spectra were measured with a Perkin-Elmer LC-480 diode array detector. Finally, a Model B220 BenchMate Workstation (Zymark Corporation, Inc., Hopkinton, USA) was used for dilution both of the samples and of the calibration stock solutions.

Materials

Iopamidol, L-N,N'-Bis(2-hydroxy-1-hydroxymethylethyl)-5-(2-hydroxypropionylamino)-2,4,6-triiodoisophthalamide (see Figure 1), was synthesized in the laboratories of SCHERING AG (Bergkamen, F.R.G.). SOLUTRAST-300 ampoules with 300 mg iodine/ml corresponding to 612.4 mg iopamidol / ml were used for calibration, for the preparation of quality control samples and for preparation of the solutions administered to the test animals.

Liquid chromatographic grade acetonitrile, water and phosphoric acid were purchased from Merck (Darmstadt, FRG).

Chromatographic conditions

The chromatographic conditions were developed in-house. Chromatographic columns of 12.5 cm x 4.6 mm i.d. were packed with Spherisorb ODS II (5 µm) by M & W Chromatographie Technik (Berlin, FRG). The analyte was eluted with a

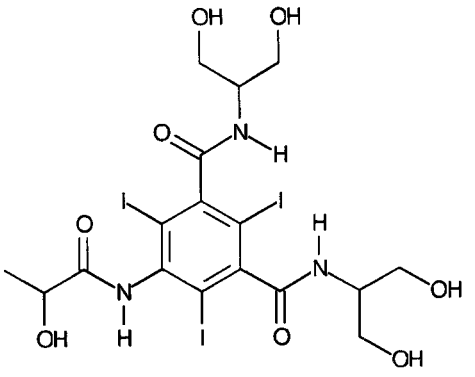

Figure 1: Structural formula of iopamidol.

gradient system. A linear gradient was used, from mobile phase A consisting of acetonitrile/water/phosphoric acid (10/990/0.2 by volume) to mobile phase B consisting of acetonitrile/water/phosphoric acid (220/870/0.2 by volume) in 10 min, the flow rate being 1.0 ml/min. Subsequently, the column was eluted with B for one min and then with A for 5 min. The column was operated at ambient temperature. The absorbance of the effluent was monitored at 240 nm. The UV spectrum of iopamidol and a representative chromatogram are shown in Figure 2 and 3, respectively.

Sample work-up (final method)

The quality control samples contained the active ingredient dissolved in physiological saline. The concentrations ranged within 0.1 and 200 mg iodine / ml.

In summary, the solutions were diluted with physiological saline to 50 µg iodine / ml. Volumes of 10 µl were injected onto a reversed phase column.

For example, in case of 2 mg iodine/ml samples, the workstation added 3.5 ml of physiological saline to a manually pipetted sample aliquot of 0.5 ml. The content of the tube was mixed by cycling (drawing and dispensing the tubes' content

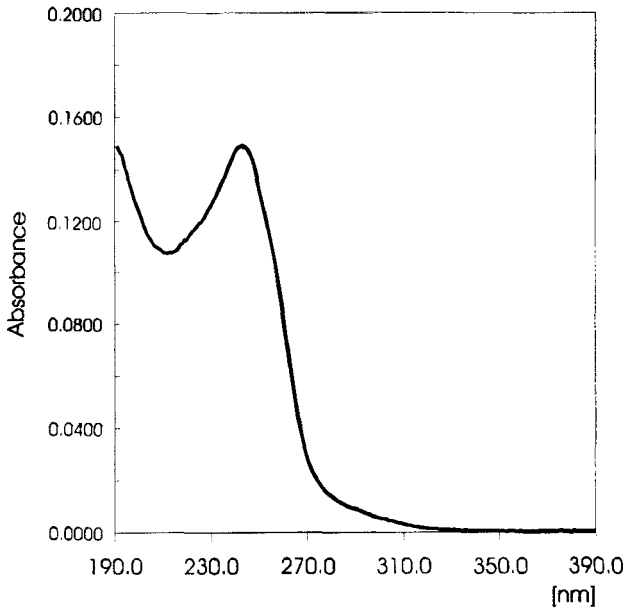

Figure 2: UV spectrum of iopamidol

The spectrum was measured on-line following chromatography of 612 µg iopamidol. The spectrum was taken at peak maximum and corrected by the background at a retention time which was shorter by 0.3 min than that of the peak maximum.

into and out of a cannula) four times. Then the solutions were volumetrically diluted with physiological saline to 4 ml, followed by another cycling. After dilution, the workstation's syringe was purged with 3 ml physiological saline. The sample work-up was followed by further dilution steps in case of higher concentrations. The BenchMate commands applied for 2 mg iodine / ml samples and the commonly used setup parameters are summarized in Table 1.

Calibration and evaluation

The six point calibration was performed by injecting amounts in the relative proportion of 100/75/50/25/12.5/6.25. 100 corresponded to 1,200 ng iodine. The

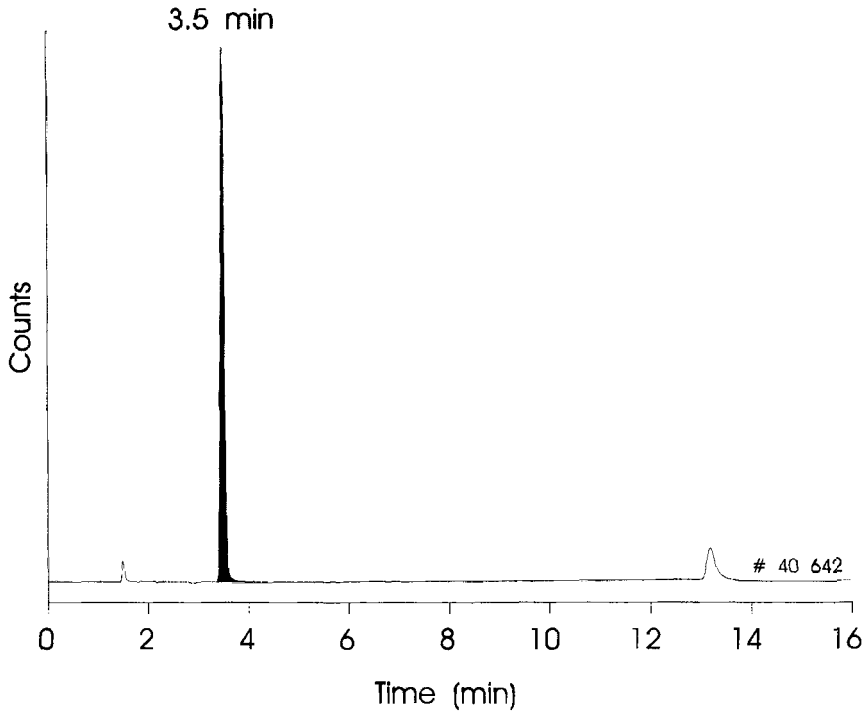

Figure 3: Chromatography of a quality control sample with 0.1 mg iodine/ml. 10 µl was injected. The peak at 3.5 min was based upon the analyte, the peak at the later retention time was related to matrix ingredients.

workstation diluted aqueous stock solutions containing 300 mg iodine/ml with physiological saline by appropriate factors. Each of the six dilutions was injected once. The calibration range was 75 - 1,200 ng iodine per injection, the volume injected 10 µl.

ACCESS*CHROM was used for evaluation of the chromatograms. The peak areas of the calibration standards were correlated with their amounts injected. The data were modelled with an unweighted linear regession (model: $y = K_0 + K_1 \cdot x$). The results were given in % of intended concentration of the samples. The intended value was always given in mg iodine / ml. The conversion factor (mg iodine -> mg iopamidol) accounts for 2.041.

TABLE 1

Listing of BenchMate Commands and Setup Parameters Applied for the Work-up of a 2-mg Iodine /ml Sample

Zymark BenchMate 2.5

Zymark BenchMate Procedure : 2MG/ML_500NG/10MML

Step 1 : Add 3.5 ml of LOESUNGSMITTEL
Step 2 : Mix by cycling 3 ml in tube 4 times
Step 3 : Dilute (volumetric) 1:5 into LOESUNGSMITTEL making 4 ml
Step 4 : Mix by cycling 3 ml in tube 4 times
Step 5 : Wash syringe with 3 ml of LOESUNGSMITTEL
Step 6 : END

SETUP PARAMETERS - 1

SAMPLE TUBE
Initial Volume: 0.00 ml

FLOW RATES

Aspirate:	0.50 ml/sec
Dispense:	1.00 ml/sec
Internal Std:	0.12 ml/sec
Mix:	1.50 ml/sec
Filter:	0.10 ml/sec
Air Push:	0.15 ml/sec

DISPENSING
Liquid driven: Y Y=YES N=NO

AUTOWASH PARAMETERS

Reagent Vol:	1.00 ml
Sample Vol:	0.20 ml

GRAVIMETRIC PARAMETERS

Gravimetric on:	Y Y=YES N=NO
Tolerance:	10%
Tare Sample Tubes:	N Y=YES N=NO

RACK PARAMETERS

LV Tube Numbering:	Y Y=YES N=NO
Reset to Sample 1:	N Y=YES N=NO

SETUP PARAMETERS - 2

LC PARAMETERS

Inject Load Vol:	0.50 ml
Calibrate every:	0 samples
Calib Replicates:	1
Calibration Reagent:	1
Calib Wash Vol:	0.00 ml

NAME REAGENTS
Reagent 1 : LOESUNGSMITTEL ; Density : 1.0000

NAME INTERNAL STANDARD
Standard : INTERNAL STD ; Density : 1.0000

BenchMate Table Setup

THE ESTIMATED TIME FOR ONE SAMPLE IS: 5.5 MINUTES.
NO FILTERS ARE USED.
NO SPE COLUMNS ARE USED.

Samples will be processed starting at Rack 1 position 1,
through position 50, then Rack 2 positions 1 through 50
or until an empty position is encountered.
Final tubes are in Racks 3 and 4.

Experimental characterization of the HPLC procedure

The analytical procedure was characterized with emphasis on limits of detection and quantitation, selectivity, linearity, accuracy, precision and ruggedness (10).

The instrumental limit of detection was determined at a signal-to-noise ratio of 2, and the linearity by injecting increasing amounts of iopamidol within the range of 75 to 3,000 ng iodine. The linearity was checked graphically to about twice the highest point of calibration in order to assure, that the calibration range does not border on the range of linearity. Accuracy and intra-assay precision were obtained by analysis of quality control samples. The inter-assay precision was estimated based upon the results of routinely analysed samples. The values of the pooled standard deviation over different concentration levels were calculated according to Scheffé (11). The squared values of the standard deviation or coeffcient of variation weighted with the degrees of freedom were summed. The square root of the resulting sum of squares divided by a sum of degrees of freedom yieided a pooled standard deviation (S.D.p). The over-all relative standard deviation (r.S.D.p.) was calculated by division of the over-all accuracy by S.D.p.

Finally, the acceptance criterion for the limit of quantitation was a coefficient of variation of $\leq$ 10% and a bias of $\leq$ 10% of expected concentration obtained following analysis of a series of real samples with decreasing concentrations.

Furthermore, the method's ruggedness was tested by investigating the influence of the dilution solvents, i. e. physiological saline and mobile phase A, and the number of dilution steps on the accuracy.

RESULTS

Characterization of the final HPLC method

The UV absorption of the column effluent was measured by a photo diode array detector yielding the spectrum shown in Figure 2. Optimal UV absorption was observed in the range of the detection wavelength used.

The system was selective for the analyte investigated as no other interfering peaks were observed. The instrumental limit of detection was 2.4 ng iodine per injection. Including an injection volume of 10 µl, the method's limit of detection was then 240 ng iodine/ml. Of course, the sensitivity could still be enhanced by factors of at least 10 by increasing the injection volumes. The linearity of the detector response was tested between 75 and 3,000 ng iodine per injection (i.e. 7.5 - 300 µg iodine/ml) and could be verified.

To determine the final method's accuracy and intra-assay precision, four quality control samples containing 0.1 - 100 mg iodine/ml were analysed yielding concentrations from 98 to 106% (see Table 2). The values of the relative standard deviation amounted to 0.4 - 1.8%. The over-all values for accuracy and precision (r.S.D.p) accounted for 102% and 1%, respectively. The limit of quantitation was defined at the lowest concentration level tested, 0.1 mg iodine/ml, because accuracy and intra-assay precision did not exceed the limits of acceptance. However, the real limit of quantitation is supposed to be lower taking into account that samples can be injected onto the HPLC column without dilution. The estimate on the basis an injection of 200 ng iodine dissolved in an injection volume of 10 µl is then 2 µg iodine/ml.

Influence of the dilution solvent on the accuracy:

The individual data of the sample work-up and the corresponding results are summarized in Table 3.

First, mobile phase A was used for the dilution of the calibration solutions and quality control samples. At the lower concentration levels, 0.1 - 2.0 mg iodine/ml, the means of the analytical results ranged from 101% to 103%. However, the higher levels of 10, 50 and 100 mg iodine/ml yielded 119% ± 0.6, 154% ± 2 and 154% ± 5. The sample work-up was performed five times. The bias exceeding 50% of expected concentration indicated that the apparatus was inaccurate where dilution factors of higher than 200 were used for dilution with mobile phase A.

For a second experiment, the solutions were diluted with physiological saline. On the average, the analytical results of the solutions with 0.1, 0.5, 2.0, 10, 50

TABLE 2

Accuracy and Intra-assay Precision of the Determination of Iopamidol in Physiological Saline

The number of replicates was five. 10 µl was injected per sample.

Intended concentration of the sample [mg iodine/ml]	**Portion injected** [ng iodine]	**Accuracy** [% of intended concentration]	**intra-assay Precision** [% of intended concentration] (standard deviation)
0.1	500	103.4	1.0
0.5	500	98.1	1.8
2.0	500	100.1	1.4
10.0	500	103.0	0.4
50	500	104.2	0.5
100	500	102.1	0.9
		Mean value: 101.8	S.D.p: 1.1

TABLE 3

Results from Testing Ruggedness Owing to Different Methods of Sample Work-up Made Manually or by a BenchMate Workstation

Concentration of the quality control samples [mg iodine/ml]	**Manual dilution** Aliquot pipetted [ml]	**Manual dilution** Final volume [ml]	**Automated dilution** - 1st step Aliquot pipetted manually [ml]	**Automated dilution** - 1st step Final volume [ml]	**Automated dilution** - 2nd step Aliquot pipetted automatically [ml]	**Automated dilution** - 2nd step Final volume [ml]	**Automated dilution** - 3rd step Aliquot pipetted automatically [ml]	**Automated dilution** - 3rd step Final volume [ml]	**overall Dilution factor**	**Analytical results** [% of intended concentration] (Mean ± standard deviation and number of replicates)
0.1	-	-	0.5	1.0	-	-	-	-	2	103 ± 1 (4)
	-	-	0.5	1.0 * (*eluent A)	-	-			2	103 ± 2 (5)
0.5	-	-	0.5	5.0	-	-	-	-	10	98 ± 2 (5)
	-	-	0.5	5.0 * (*eluent A)	-	-			10	101 ± 1 (5)
2.0	-	-	0.5	4.0	0.8	4.0	-	-	40	100 ± 1 (5)
	-	-	0.5	4.0 * (*eluent A)	0.8	4.0			40	102 ± 0.3 (5)
10	-	-	0.25	5.0	0.5	5.0	-	-	200	106 ± 2 (5)
	1.0	5.0	0.5	4.0	0.8	4.0	-	-	200	103 ± 0.4 (5)
	-	-	0.1	4.0	0.8	4.0	-	-	200	102
	-	-	0.5	5.0 * (*eluent A)	0.2	4.0	-	-	200	119 ± 0.6 (5)
50	-	-	0.5	5.0	0.5	5.0	0.5	5.0	1,000	112 ± 2 (5)
	1.0	25	0.5	4.0	0.8	4.0	-	-	1,000	104 ± 0.5 (5)
	-	-	0.02	4.0	0.8	4.0	-	-	1,000	101
	-	-	0.5	5.0 * (*eluent A)	0.09	9.0	-	-	1,000	154 ± 2 (5)
100	-	-	0.25	5.0	0.5	5.0	0.5	5.0	2,000	111 ± 1 (5)
	0.5	25	0.5	4.0	0.8	4.0	-	-	2,000	102 ± 0.9 (5)
	-	-	0.01	4.0	0.8	4.0	-	-	2,000	100
	-	-	0.25	5.0 * (*eluent A)	0.09	9.0	-	-	2,000	154 ± 5 (5)

and 100 mg iodine/ml were 103%, 98%, 100%, 106%, 112% and 111% of the intended values, respectively. The bias values at the high concentration levels were evidently lower than in the previous experiment. Thus, physiological saline should be preferred with the purposes of sample work-up. At the 50 and 100 mg iodine / ml levels, however the deviation was always higher than 10% and not yet acceptable.

Influence of the number of dilution steps on the accuracy:

For the samples of the previous experiment with 10, 50 or 100 mg iodine / ml, aliquots of 0.25 or 0.5 ml pipetted manually were diluted automatically by 3 serial steps by factors of 200 up to 2,000. The number of dilution steps was considered to be a possible source of the bias in the results. Consequently, the samples with more than 10 mg iodine/ml were prediluted manually by factors of 5 up to 50 in a third experiment to save one automated dilution step. The mean analytical results were 103%, 105% and 102% for the samples with 10, 50 and 100 mg iodine/ml, respectively, indicating acceptable accuracy values and the precise processing of the BenchMate Workstation. To confirm the results, the initially pipetted aliquot was reduced from 250, 500 and 250 µl to 100, 20 and 10 µl for the samples with 10, 50 and 100 mg iodine/ml, respectively. The dilution of the quality control samples was performed once by two steps. The over-all dilution factors remained constant at 200, 1,000 and 2,000. The results accounted for 102%, 101% and 100% of intended concentration indicating that the BenchMate worked exactly by using 2 dilutions steps.

DISCUSSION AND CONCLUSIONS

The results of the over-all validation indicate the excellent accuracy and repeatability of the analytical method. The sensitivity is sufficient. The manual method could be automated nearly completely. The preparation of the calibration stock solutions, the allocation of sample aliquots and the transfer of diluted solutions into autosampler vials remained to be done manually. Inspite of that, the saving in time accounted for ca. 25%. Problems may occur when 3 dilutions steps

are used. However, within the framework of method development the problematic BenchMate operation can be tested.

The BenchMate procedures developed for the experiments described here have already been modified for routine analyses of other compounds, i. e. other contrast media and various steroids. Microcrystalline suspensions need to be dissolved manually to prevent a possible loss in recovery. Recently, the automation of a method for analysis of test-substance diet mixtures provided for the administration to mice and rats has been tried. The BenchMate workstation added internal standard solution and extraction medium to aliquots of the test-substance diet mixture. The extraction was performed by cycling or vortexing. Because the methanolic extract can not be filtered efficiently before incorporated into the workstation's cannulas and obstructions have occured. However, partial automatization could be achieved by use of solid phase extraction. The extract were prepared conventionally as described earlier (12) and cleaned by a BenchMate solid phase extraction procedure.

In summary, the automation of our HPLC procedures yielded apparent savings in costs for routine analyses. The workstation is compact and easy-to-handle, and has found acceptance by those using it.

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