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Development and validation of an LC method for the quantitation of carbenicillin in human serum

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Abstract: An LC method for the quantitation of carbenicillin in human serum has been developed and validated. After protein precipitation with acetonitrile and evaporation, the residue was taken up by citric acid at pH 1.9. Carbenicillin and the internal standard (I.S.), piperacillin, were extracted with ethyl acetate, evaporated to dryness and reconstituted with a buffer solution. The separation of carbenicillin,, the I.S., and matrix peaks was achieved on a Microsorb C_{18}, 3 μm column with a mobile phase of acetonitrile–tetrabutylammonium–phosphate buffer (pH* 6.6). The detection was by UV at 208 nm. The run time was 8 min. The established linearity range was 0.25–20 μg ml^{-1} ($r^2 > 0.99$) with a limit of quantitation of 0.25 μg ml^{-1}. Interday precision (RSD) and bias over the entire range were 1.1–6.9% and −1.83 to +2.80%, respectively. The interday precision (RSD) and bias for the QC samples at 0.75, 3.0 and 12 μg ml^{-1} were 5.9–7.9% and −2.80 to +2.30%, respectively. Stabilities of on-system, bench top, freeze–thaw cycles and sample storage were established.

Keywords: *LC; carbenicillin; human serum.*

Introduction

Carbenicillin is a broad spectrum semisynthetic penicillin-type antibiotic and has antipseudomonal and antiproteus effects. Carbenicillin is commercially available as the sodium salt of its indanyl ester which is rapidly and completely hydrolysed to carbenicillin *in vivo* [1]. Carbenicillin is a mixture of diastereomers because it is prepared by reaction of 6-aminopenicillanic acid with a racemic phenylmalonamic acid. Carbenicillin in solution tends to be degraded even under ambient conditions [2]. Esterification of the α-carboxy group of carbenicillin helps prevent decarboxylation of the drug; therefore, carbenicillin indanyl sodium is more resistant to acid catalysed hydrolysis than carbenicillin and is generally stable in the presence of acidic gastric secretions following oral administration [1]. The chemical structures of carbenicillin and carbenicillin indanyl sodium are shown in Fig. 1.

Following oral administration of a single 764 mg dose of carbenicillin as carbenicillin indanyl sodium to healthy adults, peak serum concentrations of carbenicillin average from 6.8 to 17 μg ml^{-1} [1]. This dose is being used to treat urinary tract infections and prostatitis. A sensitive method able to determine as low as 0.25 μg ml^{-1} of carbenicillin in serum was therefore needed for the pharmacokinetic studies.

A satisfactory high-performance liquid chromatographic (HPLC) method was lacking for the analysis of carbenicillin in biological fluids. Haginaka and Wakai developed a method using precolumn derivatization with 1,2,4-triazole and mercury (II) chloride at 60°C and pH 9.0 for 10 min [3]. The limit of quantitation was claimed to be 0.1 μg ml^{-1} in serum, without data to support the claim. The lowest concentration reported was 0.5 μg ml^{-1} with a RSD% of 5.4. The chromatogram showed the carbenicillin peak being eluted on the down slope of a major interference peak. HPLC methods designed for analysing carbenicillin in preparations or bulk samples were not suitable for the analysis of serum samples due to interferences from the matrix [4–6].

Our objective in this study was to develop a sensitive, simple and reliable HPLC method for the analysis of a large number of serum samples from clinical trials. We developed a unique extraction method for penicillin-type antibiotics by combining protein precipitation and liquid–liquid extraction procedures. Serum protein was removed by precipitation

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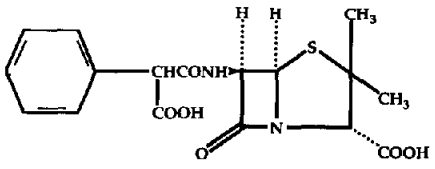
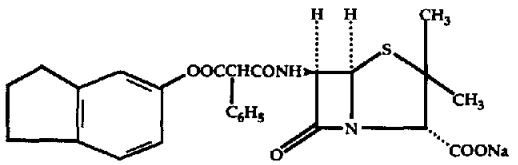
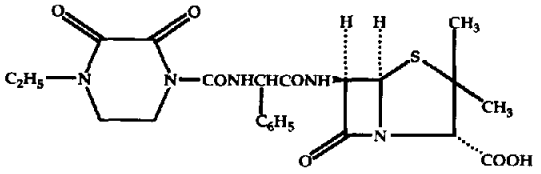


Carbenicillin



Carbenicillin indanyl sodium



Piperacillin

Figure 1
Chemical structures of carbenicillin, carbenicillin indanyl sodium and piperacillin.

with acetonitrile. The compounds of interest were extracted into ethyl acetate. Most of the interferences frequently seen with literature methods were removed by the ion-pair HPLC system developed in this laboratory. Carbenicillin and the internal standard were well separated from the matrix peaks. The limit of quantitation (LOQ) was 0.25 μg ml^{-1} which was much lower than that seen in many other published methods. The method was validated to meet the pharmaceutical industry guidelines [7].

Experimental

Materials and reagents

Carbenicillin monosodium monohydrate was from USP and the I.S., piperacillin sodium, was from Sigma (St Louis, MO, USA). The chemical structure of piperacillin is also shown in Fig. 1. Tetrabutylammonium phosphate, HPLC grade, was from Kodak (Rochester, NY, USA). All other reagents and solvents were of analytical grade and purchased from Mallinckrodt (Paris, KT, USA) or Fisher (Fair Lawn, NJ, USA). Deionized water was purified by a NANOpure® system from Barnstead. Control human serum was purchased from Worldwide Biological (Cincinnati, OH, USA).

Two primary stock solutions of carbenicillin were prepared from separate weighings, each for the preparation of standards or quality control samples (QCs). Aqueous solutions of carbenicillin primary stock and substocks were prepared at 0°C under yellow light and stored in aluminum foil-wrapped containers at −70°C. Working standards were prepared fresh daily by spiking 100 μl of 10-fold concentrated solutions which were stored at −70°C into 1.0 ml of blank control human serum. The final concentrations of carbenicillin in serum standards were 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 12.5, 15 and 20 μg ml^{-1}. Three levels of QCs, 0.75, 3.0 and 12 μg ml^{-1}, were prepared, aliquoted at 0°C, and stored frozen at −70°C.

Instrumentation

The LC system consisted of a Beckman 110B solvent delivery module, a Perkin–Elmer ISS-100 autosampler, a Waters model 481 LC UV detector set at 208 nm, and a VG® multichrom data system for VAX®/VMS. The tray of the autosampler was cooled by a Brinkman RM6 cooling system set at −10°C. The temperature at the autosampler tray was approximately 0°C to maintain the integrity of the compound. The flow rate was 1.0 ml min^{-1} and the run time was set at 8.0 min. The analytical column, a Microsorb C_{18} column of 3 μm, 10 × 0.46 cm i.d., was from Rainin (Woburn, MA, USA) and was maintained at 40°C by a Timberline column heater (Boulder, CO, USA). The mobile phase was acetonitrile–0.02 M tetrabutylammonium phosphate–0.04 M sodium phosphate monobasic (520:740:740, v/v/v). The pH of the mobile phase was adjusted to 6.6 with 10 N sodium hydroxide. The mobile phase was filtered through a 0.45 μm membrane *in vacuo* prior to use. The mobile phase was recycled.

Data treatment

Peak heights were measured using a VG® multichrom data system. The raw data output was acquired on a VG® chromserver and then transformed to the VAX®/VMS. A quadratic $1/y^2$ regression ($y = B_0x^2 + B_1x + B_2$) was used to determine slopes, intercepts and corre-

lation coefficients. The sum of the peak heights of two diastereomers of carbenicillin was used. The resulting parameters were used to calculate concentrations:

$$\text{Conc.} = \frac{-B_1 \pm [B_1^2 - 4(B_0)(B_2)]^{1/2}}{2(B_0)} . \quad (1)$$

Extraction procedures

To 1.0 ml serum sample 50 μl of piperacillin (I.S.) solution (100 μg ml^{-1}) was added. After mixing, 1 ml acetonitrile was added to precipitate proteins. The supernatant was decanted into another tube and was then flushed with a stream of nitrogen for 30 min to evaporate acetonitrile. Two millilitres of 1 M citric acid pH 1.9 was added. After mixing, 1 ml of ethyl acetate was added to extract the compounds. After vortexing for 1 min and shaking horizontally for 5 min, the organic phase was separated by centrifuging at 3000 rpm for 10 min. The organic extract was decanted into another tube and evaporated to dryness under nitrogen. The residue was reconstituted in 100 μl of 20 mM sodium phosphate monobasic aqueous solution. A 40 μl volume was injected into the HPLC. All the extraction procedures were performed in ice-water at 0°C and with protection from light.

Results and Discussion

LC Separation

Liquid chromatographic methods designed for analysing carbenicillin in preparations or bulk samples have already been reported [4–6]. When tested in this laboratory, these methods were not suitable for the analysis of carbenicillin in serum due to interference from the matrix. The method of Haginaka and Wakai for serum could not be reproduced in this laboratory with a clean background [3]. An ion-pair LC method was developed to separate carbenicillin, I.S. and interferences.

Figure 2(a)–(c) shows chromatograms of extracted blank control serum, standard at 0.25 μg ml^{-1}, and a quality control sample at 12 μg ml^{-1}. Figure 2(d) shows a chromatogram of an extracted serum collected after 6 h from a healthy volunteer dosed orally with carbenicillin indanyl sodium in a double blind study; the calculated concentration of carbenicillin in serum is 5.0 μg ml^{-1}.

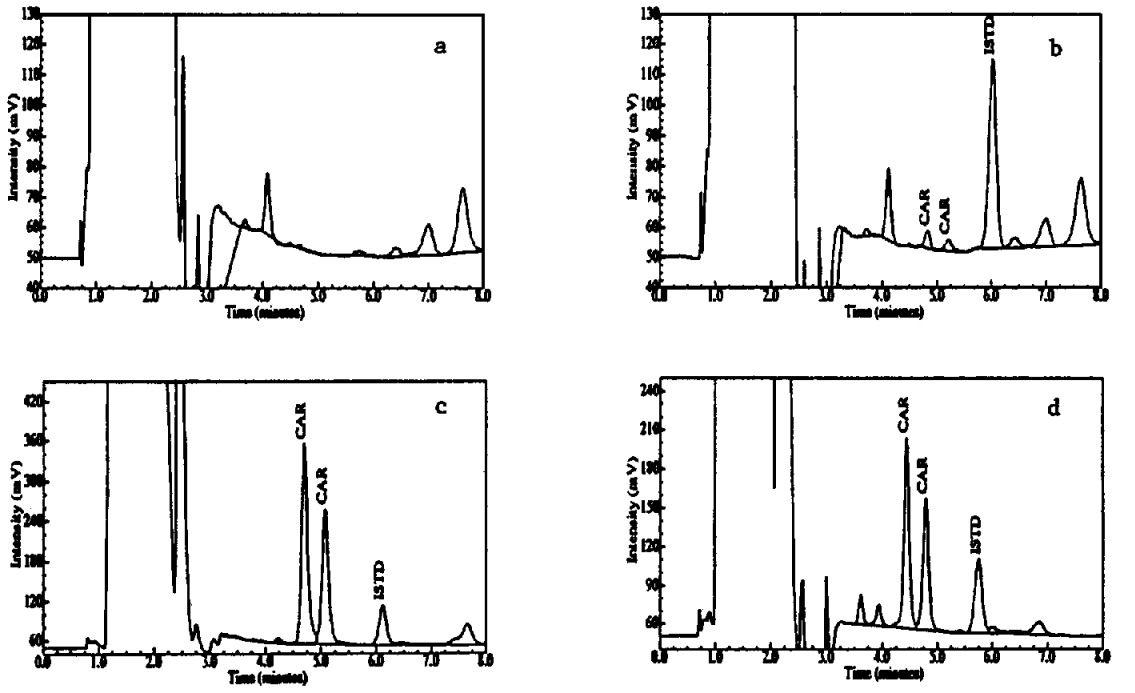


Figure 2
Liquid chromatograms of extracted blank control serum, standard, QC and a clinical sample. Peak identification: CAR = carbenicillin; ISTD = piperacillin (I.S.); a = blank control serum; b = standard at 0.25 μg ml^{-1}; c = QC at 12 μg ml^{-1}; d = a serum sample collected after 6 h from a healthy volunteer dosed orally with carbenicillin indanyl sodium in a double blind study.

The diastereomers of carbenicillin were well separated from each other and from the matrix peaks. No interference was observed in any of the eight different lots of control human serum tested. Since neither of the pure diastereomers of carbenicillin were available, the elution order and absolute configuration of the diastereomers were not determined. We were unable to coelute the two diastereomers as a single peak either by using a literature method [6] or by various combinations of different mobile phases and stationary phases (unpublished results). It has been reported that the carbenicillin diastereomers tended to elute as two peaks [4, 8]. There is evidence to suggest that diastereomers of penicillins were markedly different in their pharmacodynamic and pharmacokinetic properties *in vivo* [9–11]. With the LC conditions described in this report, the peaks of two diastereomers were well-resolved, enabling the accurate quantitation of each diastereomer at low concentration in the presence of the other at a disproportionate concentration ratio. However, it was not our purpose to investigate the biological properties of the individual diastereomers in the current study.

Since we were only interested in the total concentration of carbenicillin, the sum of the peak heights of two diastereomers was used for carbenicillin quantitation. The height ratio of the two peaks calculated from a typical curve were 1.44 (RSD% = 4.6, n = 10) for standards, 1.45 (RSD% = 5.2, n = 18) for QCs and 1.44 (RSD% = 1.1, n = 3) for clinical samples. The mean results of the area ratio of the two peaks was 1.28. Fractions corresponding to single diastereomers were collected, spiked to control blank human serum and extracted. No significant interconversion of diastereomers occurred during the extraction. On the other hand, interconversion of the diastereomers in the serum samples was very fast even at −70°C. Over 24 h, about 30% of the single diastereomer was converted to its isomer. Most clinical samples collected under 'normal conditions' may therefore consist of equilibrated isomers *in vitro*. The analytical column shows excellent stability. Identical retention times (4.6 and 5.0 min) and resolution (2.0) of the two diastereomers were obtained with a new column versus a used column of over 1000 injections. The mobile phase was recycled for up to 300 injections.

Extraction

Isolation of carbenicillin, which was free from interferences from biological fluids has not previously been reported. Extraction methods used for other penicilliins were not suitable for carbenicillin when tested in our laboratory [12–15]. Therefore a procedure for extracting carbenicillin from serum was developed. Protein had to be removed by precipitation prior to citric acid addition to avoid the formation of severe emulsion. Protein precipitation by perchloric acid yielded a lower recovery and more interference. Acidification with other acids such as hydrochloric acid and phosphoric acid resulted in much lower recovery. Back extraction of carbenicillin from ethyl acetate into an aqueous solution was attempted. The recovery was very low. Using a larger volume of ethyl acetate to extract

Table 1
Interday precision and linearity of carbenicillin standards

	Carbenicillin (μg ml^{-1})										
Std curve no.	0.250	0.500	1.00	2.50	5.00	7.50	10.0	12.5	15.0	20.0	r^2
A	0.246	0.506	1.06	2.48	4.81	7.34	10.2	12.2	15.5	NP	0.9989
B	0.248	0.506	1.01	2.46	5.05	7.48	9.85	12.6	15.1	NP	0.9999
C	0.245	0.507	1.05	2.48	5.03	7.20	9.95	12.2	15.2	20.5	0.9992
D	0.249	0.504	1.02	2.43	5.00	7.28	10.1	13.4	14.8	19.5	0.9981
E	0.254	0.486	0.999	2.48	5.14	7.60	10.0	14.2	15.6	19.3	0.9980
F	0.465*	0.501	1.00	2.43	5.08	7.47	10.4	12.2	15.1	19.9	0.9991
G	0.257	0.480	1.01	2.42	5.92	7.89	10.4	11.9	15.5	19.6	0.9977
Mean	0.250	0.499	1.02	2.45	5.14	7.47	10.1	12.7	15.3	19.8	0.9987
RSD%	1.9	2.2	2.4	1.1	6.9	3.1	2.1	6.6	1.9	2.4	0.0674
RE%	−0.07	−0.29	+2.13	−1.83	+2.80	−0.46	+1.29	+1.6	+1.71	−1.20	
n	6	7	7	7	7	7	7	7	7	5	

NP = Not performed.
* = Poor chromatogram.

compounds resulted in more chromatographic interferences.

It was necessary to perform extraction in an ice-water bath to prevent the degradation of carbenicillin. The recovery of carbenicillin from serum was 61% at 0.75 μg ml^{-1}, 62% at 3 μg ml^{-1}, and 61% at 12 μg ml^{-1}. The recovery of the I.S. was 50%.

Validation

Seven validation curves were run on seven separate days over a 3-week period. Table 1 shows the linearity and precision data at each individual standard concentration. A $1/y^2$ quadratic regression was used for standards. An unextracted standard concentration profile can be best expressed by using $1/y^2$ quadratic regressions. The peak width at the half peak height remained constant through the entire concentration range, indicating the analytical column was not overloaded. The lack of a straight line for standard concentration profile was probably due to the nonlinear response of the UV detector at the wavelength of 208 nm. Since the retention times and peak shapes of carbenicillin diastereomers and I.S. remained virtually unchanged through the study, peak height, which in general is less adversely affected by the integration than peak area, was used for the quantitation. The standards show a linear range of 0.25–20 μg ml^{-1}, with a limit of quantitation at 0.25 μg ml^{-1} for the sum of carbenicillin diastereomers (1.9% RSD and −0.07% bias). The detection limit was 0.1 μg ml^{-1} for the sum of carbenicillin diastereomers with signal-to-noise ratio equal to three.

Table 2 presents the interday and intraday accuracy and precision of QCs. The accuracy and precision data show that this method is consistent and reliable with low values of relative error and relative standard deviation for standards and QCs over the entire concentration range. The current standard curve range is suitable for most pharmacokinetic studies. Consistency on the extraction of carbenicillin and the I.S. from eight different lots of serum has been established. The results are shown in Table 3.

Table 2
Precision and accuracy of carbenicillin quality controls

	Carbenicillin (μg ml^{-1})		
	0.750	3.0	12.0
Interday			
Mean	0.767	2.92	11.7
RSD%	7.1	7.9	5.9
RE%	+2.3	−2.8	−2.1
n	40	41	42
Intraday			
Mean	0.750	2.98	12.9
RSD%	2.4	6.0	2.2
RE%	0	−0.8	+7.2
n	6	6	6

Stability

Stabilities of processing (freeze–thaw, benchtop), chromatography (on-system), and sample storage were tested and established. The data are presented in Table 4.

QCs were subjected to various cycles of freezing and thawing. The samples were then analysed. After two cycles of freeze–thaw, the values of QC samples were 104–108% of that for one cycle. After three cycles of freeze–thaw, the values of QC samples were 101–107% of that for one cycle. Benchtop stability after 2 h at 0°C was 97–103% for carbenicillin compared to the corresponding values of

Table 3
Variation of carbenicillin and I.S. extraction from eight different lots of serum

Serum no.	Spiked conc. (μg ml^{-1})	Sum of peak heights of carbenicillin diastereomers	Peak heights of I.S.	Calculated conc. (μg ml^{-1})	Relative error (RE%)
A	0.5	13923	34579	0.525	+5.0
B	0.5	14686	36285	0.528	+5.6
C	0.5	14660	36145	0.529	+5.8
D	0.5	13294	33873	0.512	+2.4
E	0.5	13116	32337	0.529	+5.8
F	0.5	14482	37697	0.501	+0.2
G	0.5	12880	34514	0.487	−2.6
H	0.5	12046	33234	0.473	−4.6
Mean		13636	34833	0.511	+2.2
RSD%		7.0	5.1	4.2	

Table 4
Stability of quality-control samples of carbenicillin in human serum

	Carbenicillin (μg ml^{-1})		
	0.75	3.0	12.0
Freeze–thaw stability (n = 6)			
1st Cycle	0.68 (4.4)	2.7 (5.4)	10.9 (4.1)
2nd Cycle	0.71 (4.2)	2.8 (3.7)	11.8 (4.1)
As % of 1st cycle	104	104	108
3rd Cycle	0.69 (5.4)	2.9 (3.1)	11.7 (4.7)
As % of 1st cycle	101	107	107
Benchtop stability (n = 6)			
0 h	0.75 (2.4)	3.0 (6.0)	12.9 (2.2)
2 h	0.75 (1.0)	2.9 (1.4)	13.3 (2.8)
As % of 0 h	100	97	103
On-system stability (n = 3)			
0–30 min	0.70 (0.2)	2.9 (1.6)	11.8 (2.4)
13 h	0.67 (6.2)	2.6 (3.6)	10.5 (1.9)
As % of 0–30 min	96	90	89
Sample storage stability (n = 6)			
Initial	0.81 (1.7)	3.0 (5.8)	11.8 (2.4)
25 days	0.75 (2.4)	3.0 (6.0)	12.9 (2.2)
As % of initial	93	100	111

RSD % are indicated in parenthenses.

normal time. The on-system stability after 13 h on an autosampler were 89–96% for carbenicillin compared to the original time. Sample storage stability was tested after QCs were stored at −70°C for 25 days. The values of the stored samples were 93–111% of the corresponding values of the original assay for carbenicillin.

Conclusions

A simple and reliable LC method had been developed for analysis of carbenicillin in human serum. The ion-pair chromatographic conditions plus the unique extraction procedure allowed carbenicillin to be well-separated from matrix interferences. As little as 0.25 μg ml^{-1} of carbenicillin in serum can be accurately quantified. The good separation of carbenicillin diastereomers enables future studies on their pharmacodynamic/pharmacokinetic properties. No degradation of carbenicillin was observed during the extraction and injection process. The analytical column showed very good stability. This method is suitable for the analysis of large numbers of samples in pharmacokinetic studies.

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