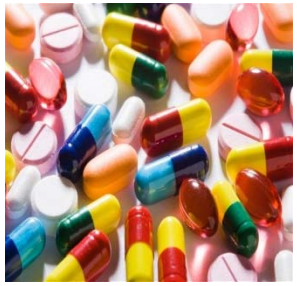
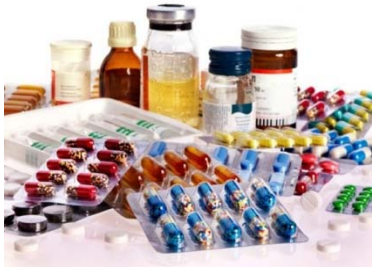


Development and validation of a simple high-performance liquid chromatographic method for the determination of curcumin in plasma

UNIVERSITI SAINS MALAYSIA

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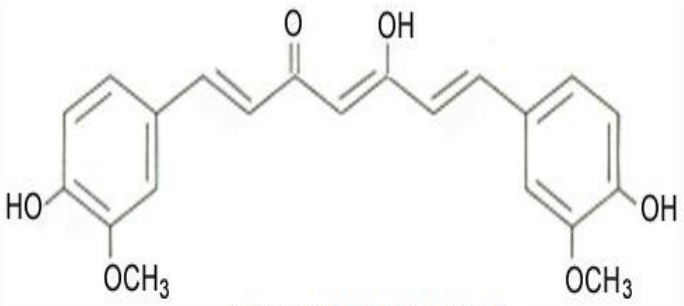
Introduction

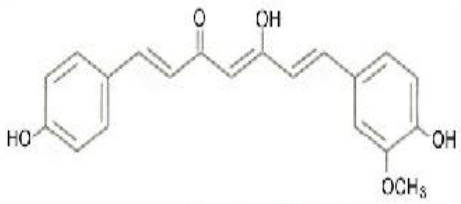
- dried ground rhizome of the perennial herb *Curcuma longa* Linn, a member of the ginger family (*Zingiberaceae*)
- regarded as the most biologically active constituent of turmeric
- also known as diferuloylmethane, or diferulylmethane
- contains turmerin (a water-soluble peptide), essential oils (such as turmerones, atlantones and zingiberene) and curcuminoids
- used as food preservative, coloring and flavoring agents
- linked with antioxidant, anti-inflammatory, anti-microbial, antiproliferative, anticancer, antidiabetic, antirheumatic, antiviral, diabetes, Alzheimer's disease, Parkinson's disease and arthritis

- 3 types of curcuminoids, namely:
  (i) bis-demethoxycurcumin (BDMC)
  (ii) demethoxycurcumin (DMC)
  (iii) curcumin [1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]

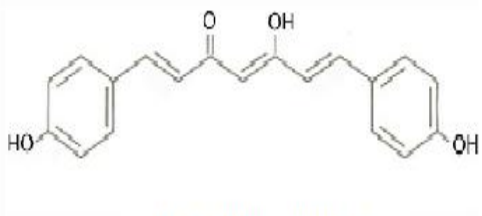
- exist in several tautomeric forms:
  (i) 1,3-diketo form (acidic and neutral conditions)
  (ii) two equivalent enol forms (alkaline medium), more energetically stable in the solid phase and in solution



Curcumin (Main Curcuminoid)



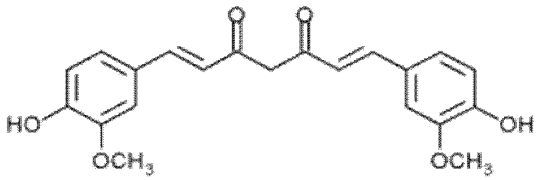
Demethoxycurcumin



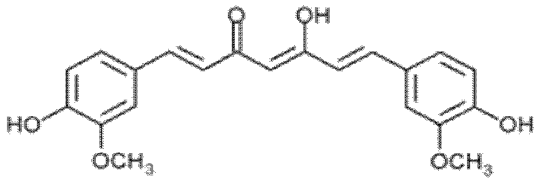
Bisdemethoxycurcumin

(Modified from: Changtam C, et al. Curcuminoid analogs with potent activity against Trypanosoma and Leishmania species. Eur J Med Chem. (2010))

Curcumin keto form:



Curcumin enol form:



Obtained from Patel AR et al., Particles comprising hydrophobic polymer and hydrophobic phenolic compound. (Publication no: WO 2012000757 A1))

(Molecular formula: $C_2iH_2o0_6$; molar mass 368.38 g/mol; bright yellow-orange powder; melting point 183°C)

Author	Title	Method	LLOQ	Sample Pre-treatment	Advantage and disadvantage
Heath *et al*. (2003)	Curcumin in plasma and urine: quantitation by high-performance liquid chromatography	HPLC-UVD	63 ng/mL	Extraction method	Long run time
Pak *et al*. (2003)	Sensitive and rapid isocratic liquid chromatography method for the quantitation of curcumin in plasma	HPLC-UVD	2.5 ng/mL	Three extraction methods	Sensitive method but large sample amount required while one of the extraction solvent is chloroform (toxic)
May *et al.* (2005)	Detection and quantitation of curcumin in mouse lung cell cultures by matrix-assisted laser desorption ionization time of flight mass spectrometry	MALDI-TOF/MS	0.04 nmol	Extraction method	Short run time and sensitive method but expensive instrument while the matrix effects are difficult to overcome
Liu *et al*. (2006)	Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin	LC-MS/MS	0.5 ng/mL	Extraction method	Short run time and sensitive method but expensive instrument, sample pre-treatment was complicated and time-consuming while the recovery is only 77.15%

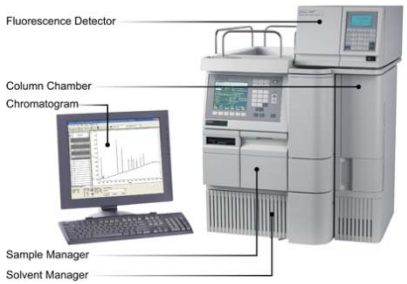
Table above shows Summary of papers reviewed

Author	Title	Method	LLOQ	Sample Pre-treatment	Advantage and disadvantage
Ma *et al*. (2007)	High-performance liquid chromatography analysis of curcumin in rat plasma: application to pharmacokinetics of polymeric micellar formulation of curcumin	HPLC-UVD	20 ng/mL	Protein precipitation method	Long run time
Yang *et al*. (2007)	Oral bioavailability of curcumin in rat and the herbal analysis from *Curcuma longa* by LC-MS/MS	LC-MS/MS	5 ng/mL		Short run time but expensive instrument
Li *et al*. (2009)	A rapid and simple HPLC method for the determination of curcumin in rat plasma: assay development, validation and application to a pharmacokinetic study of curcumin liposome	HPLC-UVD	1 ng/ml	Protein precipitation method	Very short run time, sample peak may interfered by solvent and plasma front peak while a huge amount of solvent required
Schiborr *et al.* (2010)	A validated method for the quantification of curcumin in plasma and brain tissue by fast narrow-bore high-performance liquid chromatography with fluorescence detection	HPLC-FD	0.25 ng/ml	Extraction method	Very short run time, sample peak may interfered by solvent and plasma front peak while a huge amount of solvent required

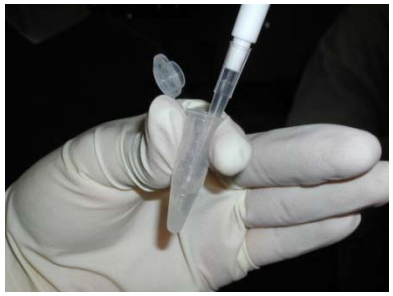
Table above shows Summary of papers reviewed

Method Development

- Various aspects should be focused on this part prior for any sample analysis.



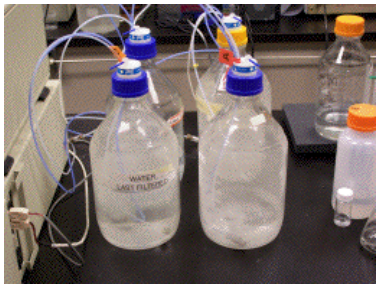
HPLC-detector Condition



Mobile Phase Condition

Method Development

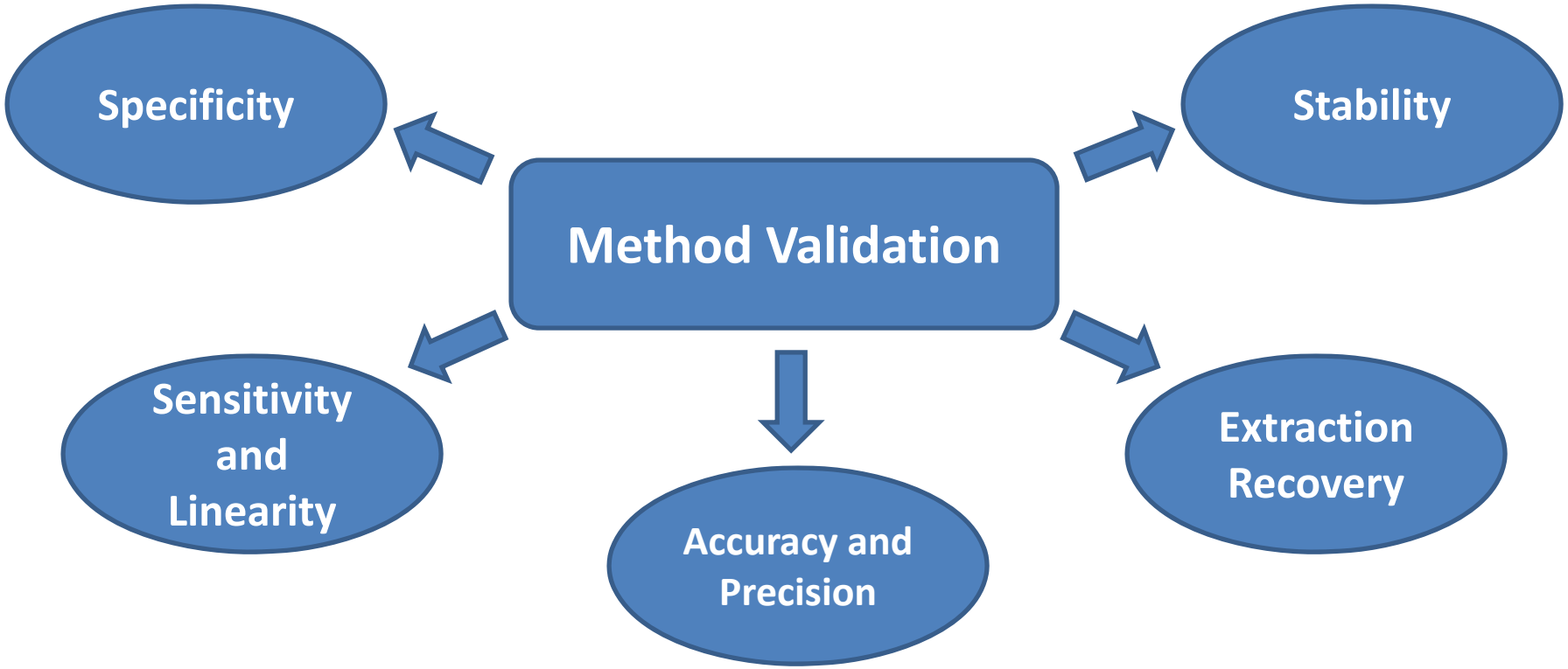
Sample Pre-treatment



Column Condition

Method Validation

- The method was validated according to the guidelines of the main regulatory agencies, such as those issued by the United States Pharmacopeia [15] and by the US Food and Drug administration [16].



Specificity

- Specificity is described as the ability of a method to discriminate the analyte from all potentially interfering substance.
- The specificity of the method was investigated by blank plasma detection, peak purity and spiking experiments with pure standard compounds.

Figure 2(a)

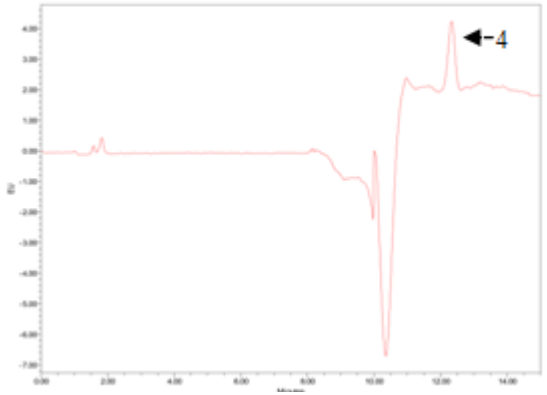


Figure 2(c)

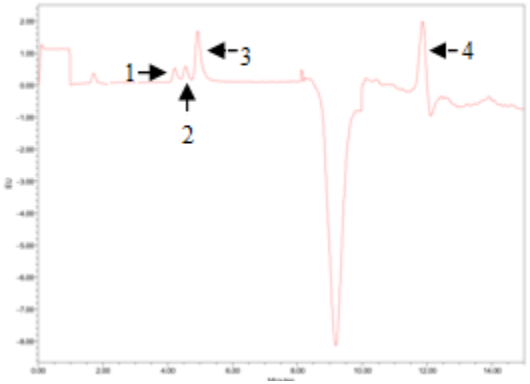


Figure 2(e)

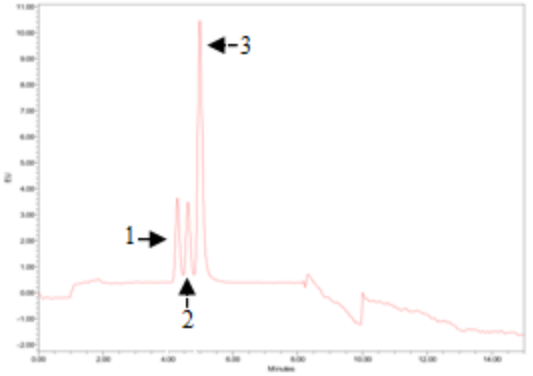


Figure 2(b)

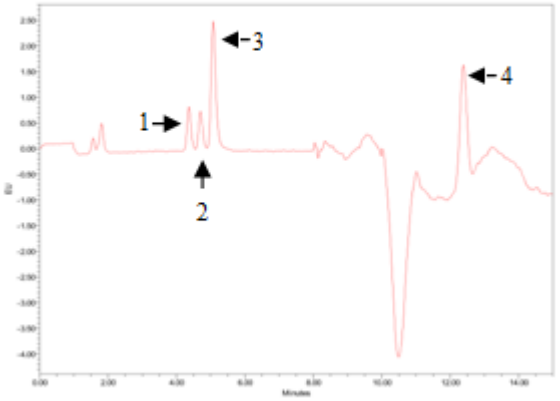


Figure 2(d)

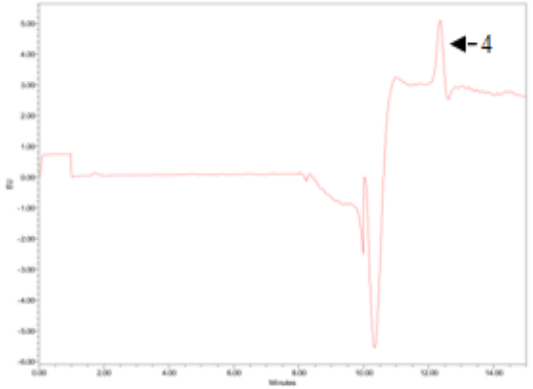


Figure 2(f)

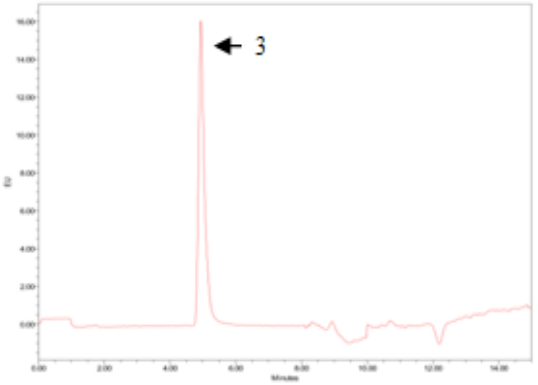
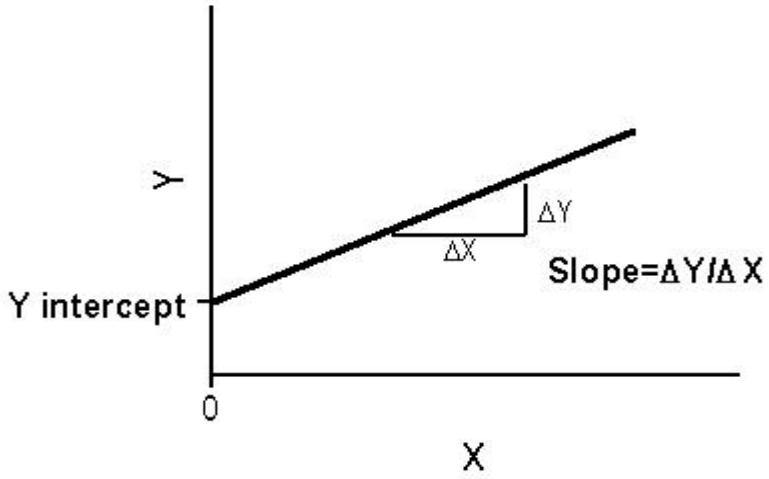


Figure 2(a). Blank plasma spiked with IS; Figure 2(b). Plasma spiked with Curcuma extract (containing BDMC, DMC and curcumin) and IS; Figure 2(c). Curcuma extract (containing BDMC, DMC and curcumin) and IS in acetonitrile; Figure 2(d). IS standard solution in methanol; Figure 2(e). Curcuma extract (containing BDMC, DMC and curcumin) in acetonitrile; Figure 2 (f). Curcumin standard solution in acetonitrile.

Peak labeled (1) BDMC; (2) DMC; (3) curcumin; (4) IS.

Sensitivity and Linearity

- The lower limit of quantification (LLOQ) is defined as the lowest concentration on the standard curve that can be quantified with accuracy within ±20% of nominal and precision not exceeding ±20% CV.
- In this study, the LLOQ was determined to be 62.5 ng/mL while the accuracy and CV were 100.41% and 1.68%, respectively.
- The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample.
- In the current study, calibration curves of plasma were linear in the range of 62.5-2000 ng/mL with $r^2 \geq 0.9999$.



Day 1	Area					Day 4	Area				
Concentration (ng/ml)	Curcumin	β-estradiol-acetate-17	Ratio	Measured Concentration	Accuracy	Concentration (ng/ml)	Curcumin	β-estradiol-acetate-17	Ratio	Measured Concentration	Accuracy
62.50	5955	361257	0.0165	62.73	100.36	62.50	5345	346627	0.0154	63.10	100.96
125.00	11949	370900	0.0322	128.26	102.61	125.00	11781	367589	0.0320	123.85	99.08
250.00	23069	361161	0.0639	260.14	104.06	250.00	24298	372270	0.0653	245.21	98.08
500.00	43411	362655	0.1197	492.71	98.54	500.00	47314	349746	0.1353	500.97	100.19
1000.00	102832	431411	0.2384	987.01	98.70	1000.00	99895	364470	0.2741	1008.03	100.80
2000.00	185684	384335	0.4831	2006.65	100.33	2000.00	190233	349292	0.5446	1996.35	99.82
		r	0.9999					r	1.0000		
		A	-5.9417					A	6.7706		
		B	4165.7281					B	3653.1123		

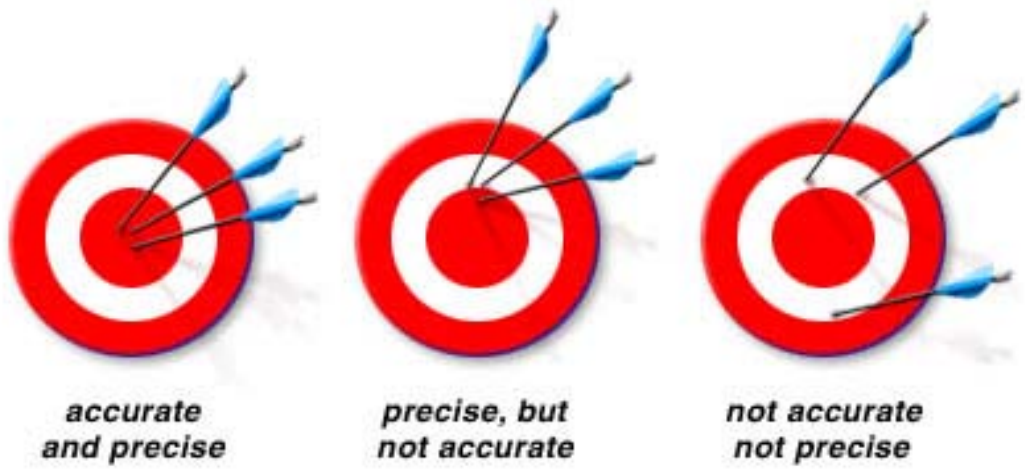
Day 2	Area					Day 5	Area				
Concentration (ng/ml)	Curcumin	β-estradiol-acetate-17	Ratio	Measured Concentration	Accuracy	Concentration (ng/ml)	Curcumin	β-estradiol-acetate-17	Ratio	Measured Concentration	Accuracy
62.50	5821	332897	0.0175	64.23	102.76	62.50	5168	329000	0.0157	60.71	97.13
125.00	13122	389053	0.0337	126.64	101.31	125.00	11804	347156	0.0340	127.73	102.18
250.00	22024	343706	0.0641	243.26	97.31	250.00	23520	347834	0.0676	250.89	100.36
500.00	42349	316921	0.1336	510.52	102.10	500.00	46146	340011	0.1357	500.39	100.08
1000.00	94574	366041	0.2584	989.87	98.99	1000.00	96571	356267	0.2711	996.25	99.62
2000.00	193544	370761	0.5220	2002.99	100.15	2000.00	191367	350837	0.5455	2001.54	100.08
		r	1.0000					r	1.0000		
		A	-2.9672					A	3.1571		
		B	3842.6876					B	3663.6761		

Day 3	Area					Day 6	Area				
Concentration (ng/ml)	Curcumin	β-estradiol-acetate-17	Ratio	Measured Concentration	Accuracy	Concentration (ng/ml)	Curcumin	β-estradiol-acetate-17	Ratio	Measured Concentration	Accuracy
62.50	5854	366751	0.0160	63.19	101.10	62.50	5596	359578	0.0156	61.47	98.35
125.00	12064	375201	0.0322	122.72	98.17	125.00	11788	359077	0.0328	125.17	100.13
250.00	24888	373249	0.0667	249.66	99.86	250.00	24386	366069	0.0666	249.82	99.93
500.00	47844	354239	0.1351	501.08	100.22	500.00	49066	369151	0.1329	494.42	98.88
1000.00	95958	353709	0.2713	1001.95	100.19	1000.00	101251	371143	0.2728	1010.53	101.05
2000.00	189521	349378	0.5425	1998.92	99.95	2000.00	183509	339864	0.5399	1996.09	99.80
		r	1.0000					r	1.0000		
		A	4.3005					A	4.0518		
		B	3676.6638					B	3689.3214		

Tables above show linearity of curcumin (n=6)

Accuracy and Precision

- The accuracy of an analytical method describes the closeness of the test results obtained by the method to the normal value of the analyte, while the precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample.
- Within- and between-day accuracy and precision (%CV) acceptance criterion for each QC was ≤ 15%.



QC sample	Nominal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy (%)	Precision (%CV)
Within-day	62.5	61.95	99.13	0.62
	125	124.37	99.5	0.74
	250	249.93	99.97	1.32
	500	495.85	99.17	0.49
	1000	996.32	99.63	0.62
	2000	1986.36	99.32	1.67
Between-day	62.5	62.81	100.41	1.68
	125	126.85	101.48	1.27
	250	255.89	102.35	0.62
	500	505.4	101.08	1.77
	1000	1007.23	100.73	1.04
	2000	2026.86	101.34	1.24

Table above shows Within- and between-day accuracy and precision of curcumin (n = 6)

Extraction Recovery

- In order to evaluate extraction recovery, a second set of plasma samples was processed and spiked post-extraction with the same concentrations of curcumin and IS that actually existed in the pre-extraction spiked samples.
- Extraction recovery for each analyte was determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to those of the samples spiked after extraction.

Recovery						
Concentration	62.50	125.00	250.00	500.00	1000.00	2000.00
1	99.62	97.91	95.94	96.65	97.63	98.77
2	98.37	96.64	97.10	98.06	100.83	100.63
3	99.21	93.29	94.70	98.06	100.08	95.23
4	99.83	96.18	96.38	98.83	98.99	89.80
5	98.50	98.24	93.62	98.29	95.69	94.05
6	97.26	98.90	95.20	98.66	95.74	98.65
Mean	98.7989	96.8587	95.4909	98.0895	98.1609	96.1879
SD	0.9536	2.0200	1.2482	0.7740	2.1787	3.9689
CV	0.9652	2.0855	1.3071	0.7890	2.2195	4.1262

Table above shows Recovery of curcumin in plasma (n = 6)

Mean of 97.26%±1.92%

Recovery						
Concentration	62.50	125.00	250.00	500.00	1000.00	2000.00
1	94.61	94.59	97.42	95.91	95.45	98.57
2	94.09	92.43	97.24	97.64	99.24	99.27
3	93.28	90.98	95.60	96.76	97.84	94.30
4	95.04	93.47	100.30	97.38	97.83	91.53
5	93.02	94.92	94.36	96.55	95.06	95.31
6	91.73	96.52	96.12	97.51	94.27	96.18
Mean	93.6283	93.8183	96.8400	96.9583	96.6150	95.8600
SD	1.2054	1.9603	2.0334	0.6711	1.9566	2.8481
CV	1.2874	2.0894	2.0998	0.6922	2.0252	2.9711

Table above shows Recovery of β-estradiol-17-acetate in plasma (n = 6)

Mean of 95.62%±1.86%

Stability

- Due to the need for occasional delayed injection or reinjection of extraction samples, the post-operative stability of treated samples in autosampler vials was assessed at ambient temperature for 8 h. Freeze-thaw stability was assessed over three cycles. QC samples were thawed at room temperature and refrozen at -20°C over three cycles and assayed. Short-term stability was evaluated to ensure that curcumin was not degraded in plasma samples at room temperature for a time period to cover the sample preparation and was assessed by exposing the QC samples to ambient laboratory conditions for 8 h.
- The long-term stability of curcumin in plasma at -20°C was evaluated by assaying QC samples at beginning, 1 month later and 2 months later.

Sample condition	Nominal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy (%)	Precision (%CV)
Freeze-thaw stability (3 cycles)	200	197.88	98.47	0.62
	2000	1999.48	100.74	1.28
Short-term stability (8 hours at room temperature)	200	201.88	100.94	2.26
	2000	2002.37	100.12	0.48
Post-operative stability (8 hours in autosampler)	200	199.7	99.85	3.46
	2000	2016.06	100.8	1.38
Long-term stability (1 month)	200	203.16	101.58	0.62
	2000	2055.98	102.8	0.97
Long-term stability (2 months)	200	196.87	98.44	1.43
	2000	2018.88	100.92	2.21

Table above shows Stability of curcumin in plasma (n = 6)

Conclusion

- A reliable, simple and sensitive high-performance liquid chromatographic (HPLC) method has been developed and validated for the determination of curcumin analysis in plasma.
- The adequate selectivity, accuracy, precision, appropriate retention time, moderate pressure and good peak shape make it suitable for our pharmacokinetic study.
- Based on the simple HPLC conditions and straightforward sample pre-treatment procedure, the current method is easy and fast to perform.
- Hence, this assay can be applied to the pharmacokinetic studies of both solubilized curcumin and its formulation in rats and so does the drug analysis in solvent as well as the drug formulation in various drug delivery systems (DDS) for future.

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thank you
danke
謝謝
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teşekkür ederim
gracias
merci
obrigado
bedankt
dziękuję
go raibh maith agat
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sukriya
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terima kasih
arigatō
grazie
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감사합니다
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obrigada
asante
хвала
tenki
murakoze
chokrane
djiere dieuf
dakon
chnorakaloutioun
gratias ago
gracies
sulpay
dekuji
mesi
madloba
najis tuke
tanemirt
trugarez
dhanyavadagalu
shukriya
merce
rahmat
xiexie
ありがとう
diolch